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THE ECOLOGY OF CHROMATIUM IN SEWAGE PONDS

Donald S. May, et al

Washington State University
Pullman, Washington

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THE ECOLOGY OF CHROMATIUM IN SEWAGE PONDS

by

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Sanitary Engineering Section

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ABSTRACT

An investigation was made into the ecological factors which influence the growth of the purple sulfur bacteria, particularly Chromatium and Thiopedia. By utilizing radioactive sulfide it was indicated that sulfide uptake is enzymatic in nature. Fructose, glucose, acetate, pyruvate, and propionate were shown to be capable of supplying electrons for the metabolism of Thiopedia. Fructose, glucose, acetate, pyruvate, lactate, formate, and propionate were able to supply electrons for the metabolism of Chromatium. These organic compounds with the exception of pyruvate were not capable of supplying carbon for synthesis of new cell material.

Thiopedia appears to be capable of utilizing substrates and producing new cell material at slightly higher rates than Chromatium. Both organisms seem to exhibit a slight preference for thiosulfate over acetate.

Concentration of sodium thiosulfate above 500 ppm does not appear to be a critical growth factor, but growth increases to a maximum value at 6000 ppm of this compound for Chromatium. Thiopedia growth levels off at a higher concentration. These concentrations are extremely high compared to those present in most natural waste effluents and may represent a limiting factor if other reduced sulfur compounds or organic intermediates are not present. It is likely that stabilization would not be possible with certain effluents.

Other environmental influences such as temperature seem to be quite critical. The optimum value is between 25° to 35°C with a rapid decline outside of this range. Light intensity is not as critical but better rates of growth were achieved at higher intensities. From studies made on growth density and dissolved oxygen it was concluded that these organisms are microaerophils rather than strict anaerobes.

In a field study made upon waste lagoons, microstratification was demonstrated to be of great importance in maintaining semi-anaerobic conditions necessary for the growth of the purple sulfur bacteria.

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ERRATA

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Page	Change
Pg 1	Reference 19 to 29.
Pg 2	Listing (1): Line three correct "Hydorgen" to read "Hydrogen". Line four underline "Chromatium".
Pg 2	Listing (3): Line three insert "could" between Chromatium and have.
Pg 2	Listing (6): Line three underline Chromatium.
Pg 20	First paragraph line three listing #2 delete "from sulfate".
Pg 20	Conclusions listing #5 change "not" to "nor".
Pg 24	Second paragraph line four change "innoculated" to "inoculated".
Pg 26	Second paragraph line four insert "and" after comma following glucose.
Pg 26	Fifth paragraph: Lines four and eight change microorganisms to microorganism.
Pg 40	Paragraph five line three change "ar" to "as".
Pg 56	Table VI to Table VII Table VII to Table VIII
Pg 60	Table X to Table VII
Pg 61	Table XI to Table VIII
Pg 61	Fourth paragraph line nine delete "was" and add "which were".
Pg 62	Table X to Table VII Table XI to Table VIII

I. GENERAL INTRODUCTION

The use of waste stabilization ponds, or lagoons, as a means of municipal and industrial waste treatment has increased rapidly in the past 20 years. The low cost of construction, operation and maintenance is responsible for their increased usage. Pond types that have been developed include "anaerobic lagoons" in which anaerobic fermentation processes predominate; "facultative ponds" in which anaerobic fermentation, aerobic oxidation, and photosynthetic reduction processes occur at varying rates; and "high rate ponds" in which oxidation and photosynthesis are balanced to yield completely aerobic stabilization and, if desired, a reclaimable excess of algae.

As a result of the work by Parker and associates in Australia (19), it was shown that increased efficiency and conservation of land area could be obtained by a two-stage process combining an anaerobic lagoon receiving high loadings followed by a larger aerobic "finishing" lagoon. Major installations of this type have been constructed, and detailed studies of this system have been carried out in the past three years at Washington State University. Daily municipal sewage loadings in excess of 1000 lb. of BOD per acre have been applied to the filled lagoons. Because the supernatant from such ponds is devoid of oxygen and rich in soluble organic matter, ammonia, and sulfides, the requirements for an adjacent aerobic pond for additional stabilization becomes necessary.

Anaerobic lagoons cannot be challenged by other types of ponds with respect to efficient land use and total BOD removal. The principal objection to such lagoons has been the odor nuisance which frequently occurs, since hydrogen sulfide and other malodorous compounds are evolved. However, anaerobic ponds at Washington State University, as well as certain other installations, (7) have not developed objectionable odors. In these ponds large populations of photosynthetic purple sulfur bacteria exist which impart a red or purple color to the water. Through the photosynthetic metabolism of these organisms much of the malodorous sulfide contained in the lagoons is presumably oxidized to elemental sulfur or sulfates. Thus the purple sulfur bacteria may act in a way to biologically deodorize the anaerobic lagoons in which they exist (6). In addition, studies indicate that two forms of the purple sulfur bacteria exist in those lagoons which develop a pink or purple color. The predominant organisms have been the large forms; Chromatium warmingii, Thiospirillum jenense and Chromatium okenii, which range in size up to sixty microns. These organisms exhibit a heterotrophic metabolism and are able to

utilize organic substrates as well as hydrogen sulfide. It seems probable that this group of bacteria may be instrumental in the removal of organic matter occurring in anaerobic lagoons.

The important contribution the purple sulfur bacteria provide in odor control and BOD removal is dimmed somewhat by the fact that the populations of these organisms are sporadic and the "blooms" which produce the vividly colored ponds occur at times of greatest sunlight and warm temperatures.

A consideration of the habitats in which blooms of Chromatium have been found poses interesting questions about the ecological requirements of this genus. Herewith is a brief summary of pertinent data on selected habitats.

(1) Five sewage treatment ponds at Pullman. Two of these were anaerobic, and all developed blooms of a large Chromatium during the summer. Hydorgen sulfide odors have been absent at all times, even when no bloom of Chromatium existed. The ponds are 1 to 1.3 m deep.

(2) Two rat waste treatment ponds near Pullman. The wastes from 6,000 to 8,000 white rats are washed into these ponds. Again, blooms develop only during the summer. The ponds are about 1 m deep.

(3) A rendering waste pond in California was studied by Cooper (7) in which concomitant blooms of Chromatium and Chlorella vulgaris occurred from February to July. However, in this case the Chromatium have been Thiopedia rosea.

(4) A petroleum refinery waste pond in California was also studied by Cooper (7). Phenol and several homologues were poured into this pond, as well as sea water. Dense populations of Chromatium occurred with the algal form, Carteria sp.

(5) Ostrau Pond was the source of Chromatium spp. (especially C. okenii) and Thiospirillum jenense for a study made by Schelegel and Pfennig (35). The pond was only about 20 cm deep, and had a dense cover of the duckweed, Lemna. Experiments showed the Lemna cover favored the growth of the Thiohodaceae. Chromatium okenii occurred mainly in the lowermost 5 cm of the water and on the surface of the mud.

(6) Lunzer Mittersee, in Austria, was studied by Ruttner (33). This lake is spring fed, 3 m deep, and is normally unstratified with a relatively constant temperature of about 6 degrees C. It is very oligotrophic, with a high rate of water flowing through it. During dry weather on two different years, this lake became stably stratified, whereupon blooms of Chromatium weissei and other Thiohodaceae developed in the lower layer of the lake. The oxygen decreased, but up to 10,000 Chromatium cells/ml were found even where the oxygen content was 3 to 4 mg/l.

(7) Krottensee, Austria, as discussed by Ruttner (33), is a meromictic lake in which a dense population of Chromatium was present at 20 to 22 m. This was also the depth at which oxygen disappeared.

(8) Beloved Lake, Russia, also a meromictic lake, is discussed by Ruttner (33) from data of Kuznetsov (33). A dense (up to 1 million cells/ml) and extremely restricted population of Chromatium developed at a depth of 14.8 to 15.0 m. This "plate" of Chromatium was only 20 cm thick. It occurred where the oxygen and H_2S curves met, at approximately zero values for each. The temperature was about 7°C. Light measurements were not made, but from other evidence Ruttner concluded that the light concentration must have been far below that necessary for algal photosynthesis.

Five environmental factors in these habitats show especially interesting contrasts. These are: depth, light, temperature, hydrogen sulfide, and dissolved oxygen. Thus, the depth at which blooms have been found range from 20 cm to 22 m. Moreoever, the vertical range of depth is sometimes extremely restricted. Light measurements were not made in any of the habitats described, but the variations in depth (and in the case of the Ostrau Pond, cover by floating vegetation) indicate great differences in the quantity and quality of light available to these bacteria. Temperature varies from 6 to about 30°C or more. In view of the abundance of Chromatium in cold water, even when it is near the surface (as in Lunzer Mittersee), it is puzzling that blooms of Chromatium in the ponds in the Pullman area developed only during the summer. Oxygen was presumably low or absent in most of the habitats, yet thriving populations of Chromatium occurred in Lunzer Mittersee at 4 mg oxygen/ml. Hydrogen sulfide was usually not detectable during blooms of Chromatium, presumably because the bacterium was utilizing it as rapidly as it was produced. Yet why did Chromatium occur in Lake Beloved only where H_2S was near zero? At greater depths the H_2S concentration increased. (Possibly light was a limiting factor.)

More precise measurements of H_2S concentrations would be desirable, and also measurements of the quantity and quality of light in various parts of the spectrum, including the short infrared. Also, sampling to determine whether sharp microstratification might sometimes exist in sewage ponds would be worthwhile, in view of the sometimes extremely restricted vertical distribution of Chromatium.

The purpose of this study, therefore, was to extend the knowledge concerning the ecological factors which influence the growth of the purple sulfur bacteria, particularly the large variety of Chromatium, in anaerobic stabilization lagoons.

Specifically the aims of this project were:

1. To isolate species of the Thiorhodaceae which exist in stabilization lagoons now being studied by this research group.

2. To study, by pure culture techniques, the physiology of the large species of Chromatium and Thiopedia with respect to metabolism of inorganic and organic sulfur compounds, volatile acids, and other metabolic intermediates found in anaerobic lagoons.

3. To study by the use of laboratory cells into which populations of Chromatium and Thiopedia were introduced several ecological factors related to these organisms. These factors included light, temperature, dissolved oxygen, and reduced sulfur compounds. This was done in an attempt to determine the relationships of these ecological factors to the propagation of the organisms.

4. To make continuous observations of lagoons receiving municipal sewage, and animal wastes so that the physical and chemical factors contributing to "pink or purple" ponds may be studied.

II. LABORATORY STUDIES

A. Isolation and Culture of Species of Thiorhodaceae

The purple sulfur bacteria are flagelated, rod-shaped or ovoid organisms belonging to the family Thiorhodaceae of the order Pseudomonadales. These organisms contain green bacteriochlorophyll and also have a high concentration of carotenoid pigments. The later pigments mask the green color of the bacteriochlorophyll and give the organism its purple hue. The organisms store sulfur internally and produce sulfate with age.

The purple sulfur bacteria are unique in many ways. They are anaerobic or microaerophilic photosynthetic bacteria that normally use hydrogen sulfide as the electron donor. The reaction may be represented by the following equations:

$$CO_2 + 2H_2S \xrightarrow{\text{light}} (CH_2O)x + 2S^o + H_2O$$

$$2H_2O + 2CO_2 + H_2S \longrightarrow 2(CH_2O)x + H_2SO_4$$

Preliminary studies by the research group indicated that the ponds producing a purple "bloom" contained two forms of Thiorhodaceae; a small form of the genura Chromatium and a larger form of the genura Thiospirillum. The larger form, which may range up to sixty microns in length, usually predominates and has been found to metabolize organic compounds as well as hydrogen sulfide. It is believed that these organisms materially contribute to the BOD reduction in the anaerobic ponds as well as providing hydrogen sulfide control.

The initial objective of this research proposal was to isolate the species of Thiorhodaceae contained in the experimental lagoons. Several types of enrichment media have been utilized for the isolation of the purple sulfur bacteria. Van Niel (44) developed a medium which has been used by other investigators; Cooper (7) and Schlegel and Pfennig (34). A medium described by Pfennig (32) has been used with good success in the culture of the red and green sulfur bacteria. Cooper (7), in his studies of ponds receiving petroleum wastes, found that a basal medium receiving an addition of sterile pond fluid seemed to provide growth factors needed by the organisms. However, in all these instances, species of the small Chromatium and Thiopedia were obtained. Schlegel and Pfennig (35) described an enrichment medium which was quite successful in fostering growth

of the large species of Chromatium okenii. This medium consisted of a basal mixture of inorganic compounds which was mixed with varying concentrations of garden earth or clarified mud. It was found that optimum growth was obtained when 20-35% of the basal medium was mixed with 65-80% of the earth.

For the microbiological studies, various media were inoculated using the pour plate technique in an effort to obtain systems suitable for the enumeration and isolation of chemosynthetic and photosynthetic anaerobes. Among the media used for chemosynthetic anaerobes were Brewer's Thioglycollate Agar, Nutrient Agar, Brain Heart Infusion Agar, Tryptic Soy Agar, Plate Count Agar, and Casitone Agar. These media were all products of the Difco Laboratories.

An approximation of the numbers of Thiorhodaceae and Athiorhodaceae was made using media designed for the particular physiological requirements of the two families. The members of the former family were assumed to be capable of growth in the absence of preformed organic materials (autotrophic), while members of the Athiorhodaceae were assumed to require preformed organic materials (heterotrophic). A basal medium (medium A) described by Skerman (37) which contained only mineral salts was used to estimate the Thiorhodaceae. The Anthiorhodaceae were estimated by the increase in photosynthetic colonies appearing in the basal medium supplemented with 0.2% yeast extract and 0.2% peptone (medium B) given by Van Niel (46).

Medium A		Medium B
Compound	g/l	
NH_4Cl	1.0	
KH_2PO_4	1.0	Same as medium A plus 2 g/l peptone and yeast extract.
$MgCl_2 \cdot 6H_2O$	0.05	

Medium A		Medium B
Compound	g/l	
$NaHCO_3$	2.0	Same as medium A plus 2 g/l peptone and yeast extract.
$Na_2S \cdot 9H_2O$	1.0	
Agar	15.0	

Freshly prepared solutions of sodium bicarbonate and sodium sulfide were autoclaved separately and added aseptically to the sterile media.

Carbon dioxide was always bubbled through the cooled sodium bicarbonate before adding to the media. The pH of each medium was approximately 7.5. Plates inoculated for counting purposes were incubated for 11 days in a Model 3640-2 National Appliance Co. anaerobic incubator in an environment of 95% nitrogen - 5% carbon dioxide gas mixture. Throughout the incubation period, anaerobiosis was maintained and checked by the reduced condition of alkaline methylene blue solution placed in the incubator. Light for photosynthetic activity was supplied by a 150-watt light bulb located outside the window of the incubator. The incubation temperature was maintained at 28°C.

Direct counts of one of the photosynthetic organisms (later identified as Thiopedia by C. B. Van Niel) present in the ponds were routinely used because of its poor growth on the two previously described media. The direct counts were made using a No. 900A Petroff-Hauser bacteria counting chamber.

Isolation procedures for most species involved transfers from single colonies on the plates to shake tubes containing A or B medium, depending on the source of isolation. Single colonies were then picked from the shake tubes and passed through three successive transfers to insure purity. Stock cultures were maintained on the same medium. Isolates that grew poorly on the above media were grown on a chemically defined medium (C) described by Larson (23). Solutions containing sodium thiosulfate, sodium sulfide, and sodium bicarbonate were autoclaved separately and added aseptically to the sterile medium.

The isolates were further studied in liquid cultures prepared in glass-stoppered bottles completely filled with the medium and incubated at 28°C at a distance of 30 cm from a 150-watt incandescent light bulb. A 10% inoculum of actively growing cells, in the same medium, was used for all growth experiments. The extent of growth was measured by visual observation or, when quantitative measurements were required, by total nitrogen content of washed cells as described by Umbreit et. al. (42).

Medium C

Compound	g/l
NH_4Cl	1.0
KH_2PO_4	1.0
$MgCl_2 \cdot 6H_2O$	0.5
$CaCl_2$	0.4
Ferric citrate	0.7

Trace Salts

Compound	mg/100 ml
H_3BO_3	280
$MnSO_4 \cdot 9H_2O$	210
$CuSO_4 \cdot 7H_2O$	20
$Na_2MO_4 \cdot 2H_2O$	75
$CoSO_4 \cdot 7H_2O$	45

$Na_2S_2O_3 \cdot 5H_2O$	3.0	$ZnSO_4 \cdot 7H_2O$	25
$Na_2S \cdot 9H_2O$	0.1	Versene	100
$NaHCO_3$	5.0		
Trace salts	2.0 ml		

In all, 14 morphologically different isolates were obtained from several hundred colonies examined over a 2-year period. These were arbitrarily separated into three groups. The first group was composed of nomotile cocci containing sulfur granules. These measured from 2 to 4 μ in diameter and were characteristic of the genus Thiopedia. The second type consisted of motil rods from 3 to 6 μ in length with visible intracellular sulfur granules. These were tentatively identified as Chromatium. Those in the third group could be described as motile and nonmotile rods that were generally less than 2 μ in length and contained no visible sulfur granules.

Although Thiopedia were evident in the pond throughout the year, they were found in greatest numbers during the summer months. Organisms in the second group appeared in greater numbers in samples collected in the spring (April-June) and fall months (September-November). Two isolates were characteristic of the second group and were designated as Chromatium I and II. Organisms characteristic of the third group were observed in the ponds at all times during the winter months. Eleven of the isolates were of the type described in the third group.

The Thiopedia T_1 isolate was obtained in shake tubes containing A and B media after a 20-day incubation period. The colonies were small and failed to grow when transferred to liquid cultures of the same media. Growth in liquid cultures was obtained in Larson's chemically defined medium (C), when supplemented with sterile filtered waste. Once growth was obtained in the supplemented medium, a larger inoculum was used to initiate growth in Larson's medium without waste supplement. Further subcultures grew well in liquid and solid preparations of this medium. All 14 isolates of the other organisms were later found to grow in Larson's medium.

B. Sulfide Uptake By Chromatium Sp.

Research indicates that different species of purple sulfur bacteria have varying abilities to oxidize different sulfur compounds. Growth experiments and short-term experiments in a stirred cuvette have shown that Chromatium okenii, strain Ostrau, is able to oxidize only sulfide and elementary sulfur; while thiosulphate, sulfite, and thioglcollate cannot be utilized as reducing agents for photosynthesis (42).

It was the purpose of this investigation to determine if metabolic uptake of sulfide occurred in an unidentified species of Chromatium, a

purple sulfur bacterium isolated from a local sewage lagoon. The study was facilitated by utilization of radioactive sulfide, as sodium sulfide, as a tracer material. The results of this investigation were used to determine the feasibility of further use of radioactive sulfide in the study of the metaboli m of this particular species and other species of purple sulfur bacteria.

Experimental Theory

Cells of Chromatium sp. were placed in a medium containing only radioactive sodium sulfide as the sulfide source. If the cells placed in this medium contain radioactivity in an amount greater than could be attributed to absorption on the cell surfaces, uptake of sulfide by the cells would be implicated.

If uptake occurs, it would be interesting to determine if the uptake were active (an enzymatic process) or if it were a simple diffusion across the cell membrane. If the prior mechanism were in effect, it would probably indicate that the organism has importance in sulfide removal from the lagoon.

One method to differentiate between active transport and diffusion would be to run experiments at room temperature (25°C) and in a cold room at 4°C. If there were significant differences in the rate of uptake, this could be assumed to be a result of a slowing of the enzymatic processes at the colder temperature. Temperature differences would not affect the rate of diffusion into the cell.

The Chromatium sp. will grow in a mineral salts medium with or without organic material, although better growth is obtained with organic material present. It will not grow in a medium containing organic material without free sulfide included.

When the organism is growing in the lagoon, an abundance of organic material and reduced sulfur compounds is present. A medium containing organic material was usedin this experiment in order to simulate natural conditions.

Materials and Methods

Organism

The purple sulfur bacterium Chromatium sp. used in this study was isolated from a local anaerobic lagoon which received the liquid wastes from a white rat farm. Cultures of this organism were grown in a liquid medium under anaerobic conditions. Light for growth was obtained from a 100-watt

tungsten filament light bulb. Growth required from 3 to 5 days at an incubation temperature of 30°C. Stock cultures were also grown on agar slants of the medium and stored at refrigerator temperatures.

Media

The basal medium for growth of the organism contained (g/l): yeast extract, 2.0; peptone, 2.0; NH_4Cl_2, 0.1; KH_2PO_4, 0.5; $Mg\ Cl_2$, 0.2; NaCl 0.5.

The following supplements were added as sterile solutions to the autoclaved base medium (g/l): $NaH\ CO_3$, 0.5; $Na_2S \cdot 9H_2O$, 0.5. Five per cent solutions of $NaHCO_3$ were prepared and sterilized by filtration through a Seitz filter. Five per cent solutions of $Na_2S \cdot 9H_2O$ were prepared and sterilized by autoclaving.

Growth of Cultures

The organism was innoculated into screw top test tubes containing 10 mls. of the base media, including the supplements. The organisms were incubated under anaerobic conditions at 30°C, receiving light from a 100-watt tungsten bulb.

Isotope

Five millicuries of sulfur ^{35}, as sodium sulfide, was acquired from Nichem, Inc.; its nuclear and chemical composition was as follows: $Na_2S^{35} \cdot 9H_2O$; .042 grams; 5 mc; 11.1 mc/mM/ .168 Mev B^-; 86.7 day half life; the isotope was dated 9-14-64. The radioactive sodium sulfide was dissolved from the glass vial in which it was shipped, using 100 mls of deionized double-distilled water. The isotope was placed in a plastic container and refrigerated until needed. This stock solution contained 1.85×10^6 dps/ml. Concentration sulfide in the sample obtained was calculated to be:

$$\frac{11.1 \text{ mc}}{1 \text{ mM}} = \frac{5 \text{ mc}}{x \text{ mM}} \qquad x = .45 \text{ mM}$$

The isotope was diluted in 100 mls of stock solution. If 1 ml of this was placed in 100 mls of medium it would equal a $\frac{1}{10,000}$ dilution. Thus, 1 ml of medium would contain .000045 mM = .045 uM of sulfide.

Uptake of Sulfide

Experiments were carried out at room temperature and in a cold room at 45°C.

1. Anaerobic Growth Vessel: The growth vessel was a modified Erlenmeyer Flask with a side arm. The side arm was located to facilitate easy removal of the vessel contents with a hypodermic syringe. Placement of a vaccine cap over the end of the side arm maintained anaerobic conditions during sampling. A photograph of the vessel is shown in Figure 1. An appropriate amount of medium was placed in the vessel such that after addition of the cells and supplements, the final volume was 100 mls. The vessel was provided with a plastic coated magnetic stirrer and autoclaved. After it had cooled, 2 mls of $NaHCO_3$ were placed in the vessel, and carbon dioxide gas was bubbled through the medium for 20 minutes to provide anaerobic conditions.

Figure 1. -- Anaerobic Vessel

2. Preparation of Cells: Ten ml. portions of nonradioactive sulfide-containing medium were used to grow the organisms initially. The organisms were harvested by centrifugation upon reaching the logarithmic growth phase, this being determined by a Photoelectric colorimeter. The cells were then washed twice and resuspended in 9 ml. portions of the medium without sulfide. This eliminated most of the sulfide that was clinging to the cell surfaces from the growth medium.

3. Sampling: The cells, suspended in 9 mls. of medium without sulfide, were then injected, via syringe, into the vessel. With the light on, the cells were allowed to equilibrate for 10 to 15 minutes. Then the specified amount of isotope was injected into the already mixing medium in the vessel. A "zero time"* sample of 1.5 mls. was taken with a syringe,

* Term means the sample was taken after a short mixing time of several seconds.

immediately following injection of the isotope. Samples were removed at 20 to 30 second intervals for the first two minutes and at varying intervals up to one hour, for a period of 1 to 6 hours.

Immediately after the samples were taken, they were filtered through a Millipore membrane, having a diameter of 25 mm and a pore size of 0.45 μ. The organism was collected on the filter, and the filter was placed on a planchet and allowed to air dry. The planchet's interior was previously coated with rubber cement to minimize warping of the filter surface. The filtrate, when saved, was collected in a test tube after passing through the filter. Filtrate samples of 0.1 or 0.2 ml. were air dried on a planchet. The experimental equipment is shown in Figure II. The filter and filtrate samples were placed in a desiccator until ready for counting. Samples were counted for 3 minutes each on a Tracerlab, Inc., Multi-Matic gas flow proportional counter. These counts were corrected for background radiation.

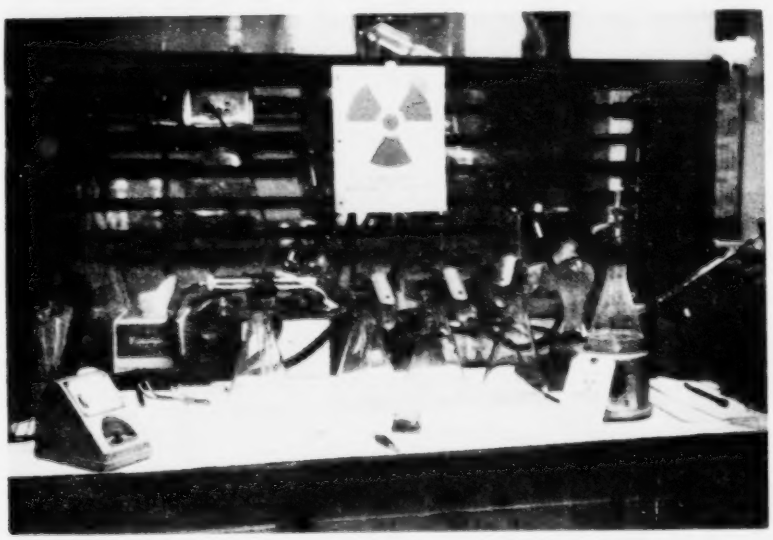

Figure 2. Experimental Equipment
Displaying the Method of Sampling and Injection

All glassware that came directly in contact with the isotope had previously been coated with a liquid silicon product to lessen absorption of the isotope onto the glass surfaces.

A photoelectric colorimeter reading was made of cell concentration before and after the experimental runs to determine if there was any significant increase in cell concentration over the initial reading.

A "zero time" sample was obtained to correct for the radioactivity absorbed onto the surface of the cells and the filter. Any increase in radioactivity above the "zero time" sample would be assumed to be due to uptake by the organism.

Preliminary Investigations

Preliminary investigations were undertaken to determine the following: (a) the amount of cellular material needed to detect uptake, (b) the feasibility of the proposed experimental procedure, and (c) the amount of isotope needed.

Experiment one used two mls. of the stock isotope solution. This amount was expected to provide enough sulfide for continuous uptake by the cells throughout the experimental period. Each ml. of medium contained approximately .09 μM of sulfide. A cell concentration of 10 units was detected by the photoelectric colorimeter. The results of experiment one are shown in Figure 3. From the results, it appeared that too few cells were used. Uptake of sulfide by the cells was indicated. The rate of uptake in the first hour was greater than the rate of uptake in any of the next five hours, thus cellular rate of uptake diminished with time. This indicated that the cells were probably near saturation with sulfide within the first hour, and that a greater concentration of cells should show a more rapid rate of uptake.

The second experiment was designed to determine the effect of a higher cell concentration on the rate of uptake. Results of this experiment are shown in Figure 4. The greater concentration increased the rate of the sulfide uptake. It was determined from this experiment that the optimum concentration of cells was about 30 units, as determined by the photoelectric colorimeter.

Tabulated results for Figures 3 and 4 (runs 1 and 2) may be found in the Appendix.

Results of the preliminary investigations were: (a) an uptake of sulfide by the cells occurred, (b) and increase in the cell density to 30 units increased the rate of sulfide uptake, (c) 2 mls. of isotope were adequate to detect sulfide uptake, and (d) the procedure used in the preliminary experiments was feasible.

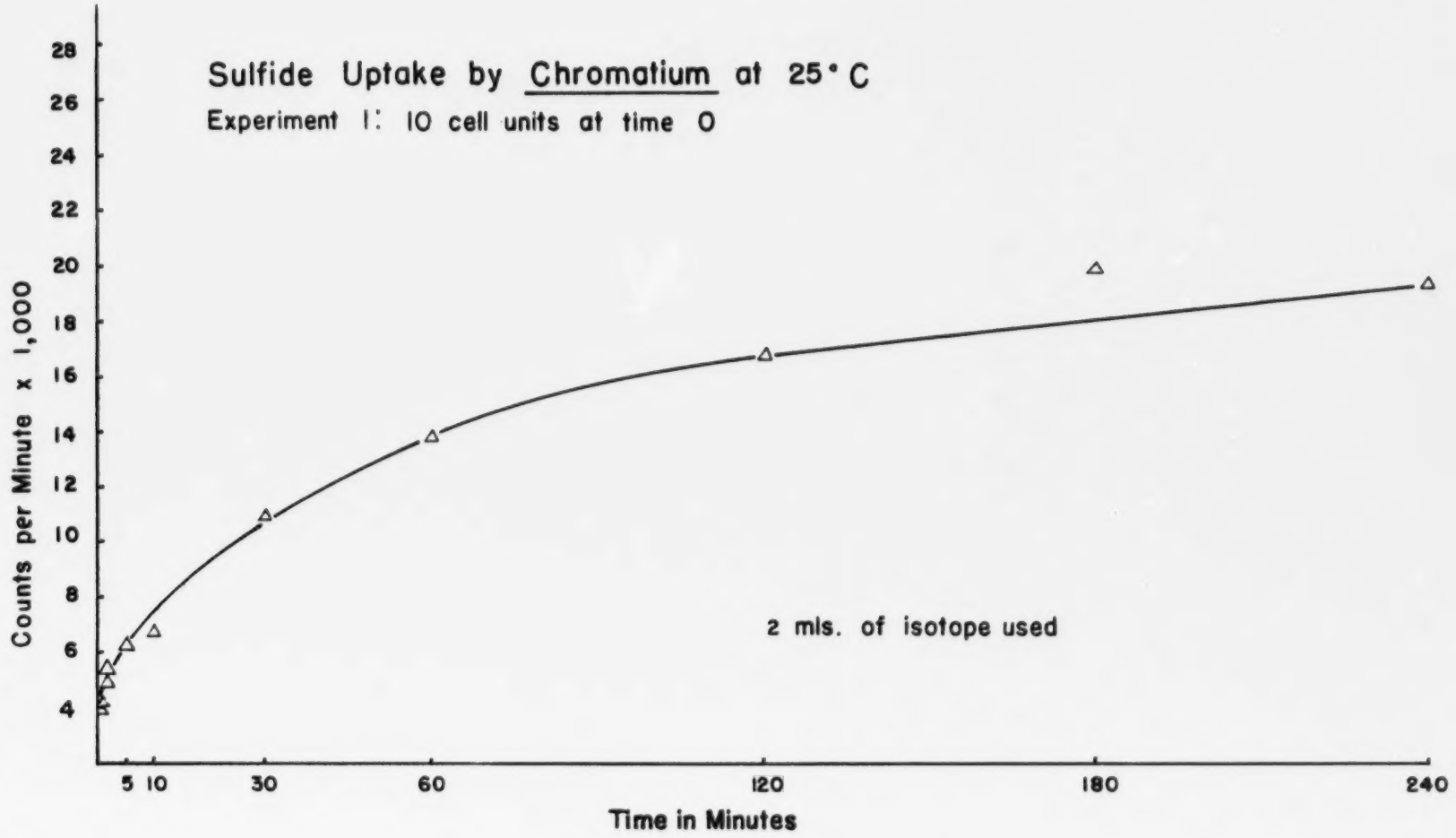
Sulfide Uptake by Chromatium at 25° C
Experiment 1: 10 cell units at time 0
Counts per Minute x 1,000
28
26
24
22
20
18
16
14
12
10
8
6
4
2 mls. of isotope used
5 10
30
60
120
180
240
Time in Minutes

Fig. 3

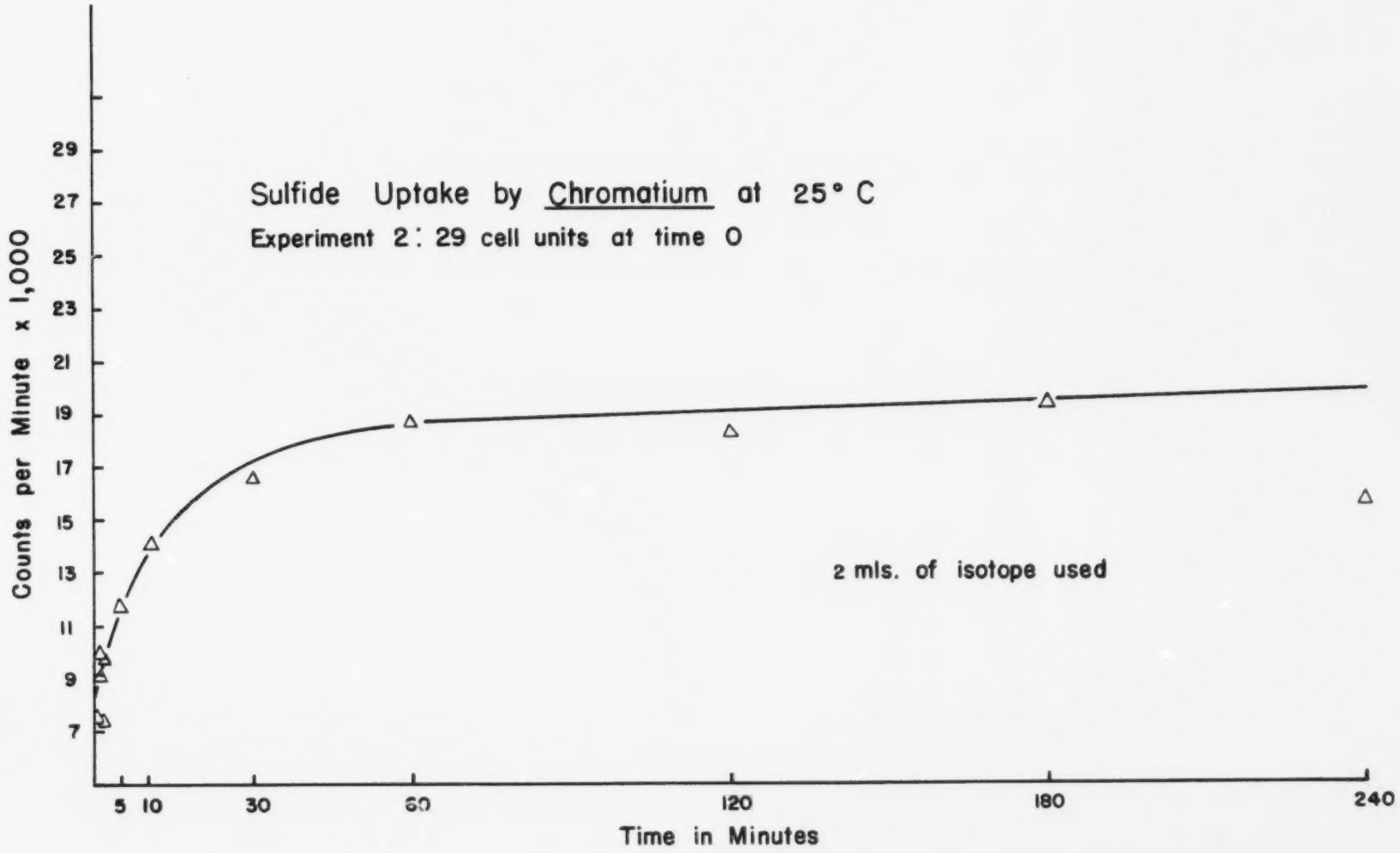
Sulfide Uptake by Chromatium at 25° C
Experiment 2: 29 cell units at time 0
Counts per Minute x 1,000
29
27
25
23
21
19
17
15
13
11
9
7
2 mls. of isotope used
5
10
30
60
120
180
240
Time in Minutes

Fig. 4

Procedure

In order to determine if a greater concentration of sulfide would increase the rate of sulfide uptake, it was decided to increase the amount of isotope to 5 mls. Also to be determined was whether the region of rapid uptake would be extended over a greater period of time, due to the greater sulfide concentration.

To differentiate between active enzymatic uptake and simple diffusion into the cells, simultaneous runs were made at room temperature (25°C) and in a cold room (4°C).

Cells for these runs were mixed together, with each anaerobic flask receiving approximately half of the cells. An effort was made to place a cell concentration of approximately 30 units in each flask, as measured by a photoelectric colorimeter.

Before the experiment was begun, the anaerobic vessel and the cells therein were allowed to equilibrate for an hour in the cold room.

In both the cold room (4°C) experiments, a lesser volume of medium was used. This resulted in a higher specific activity, as indicated by the higher count rates of the "zero time" samples.

Results

The results of the first dual experiment (runs 3 and 4) can be seen in Figure 5. The rate of sulfide uptake by the cells was reduced in the 4°C sample.

Using approximately the same conditions, the previous dual experiment was repeated for comparative purposes.

The results of the second dual experiment (runs 5 and 6) are shown in Figure 6. The results are similar to those obtained in the first dual run although some of the samples are out of range on Figure 6. Samples giving the high reading were found to contain small rubber particles from the vaccine cap.

Most of the experiments were carried out over an extended period of time, but only the first four hours are represented in Figures 3 through 6. Tabulated results for runs 3 through 6 may be found in the Appendix.

Discussion of Results

Enzymatic processes are affected by temperature difference, while simple diffusion processes are not. An enzymatic process will operate

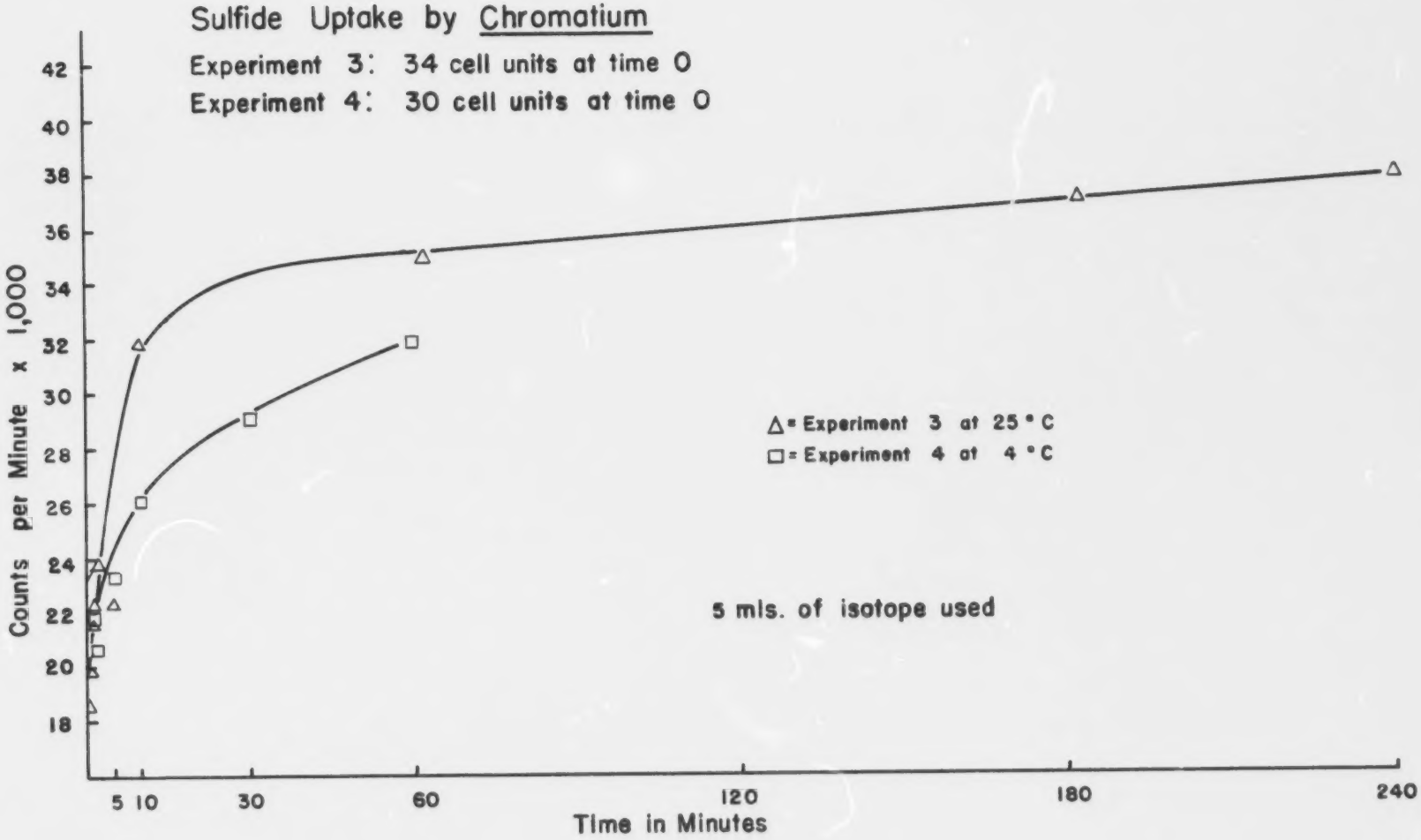
Sulfide Uptake by Chromatium
Experiment 3: 34 cell units at time 0
Experiment 4: 30 cell units at time 0
Counts per Minute x 1,000
42
40
38
36
34
32
30
28
26
24
22
20
18
△ = Experiment 3 at 25 °C
□ = Experiment 4 at 4 °C
5 mls. of isotope used
5 10 30 60 120 180 240
Time in Minutes

Fig. 5

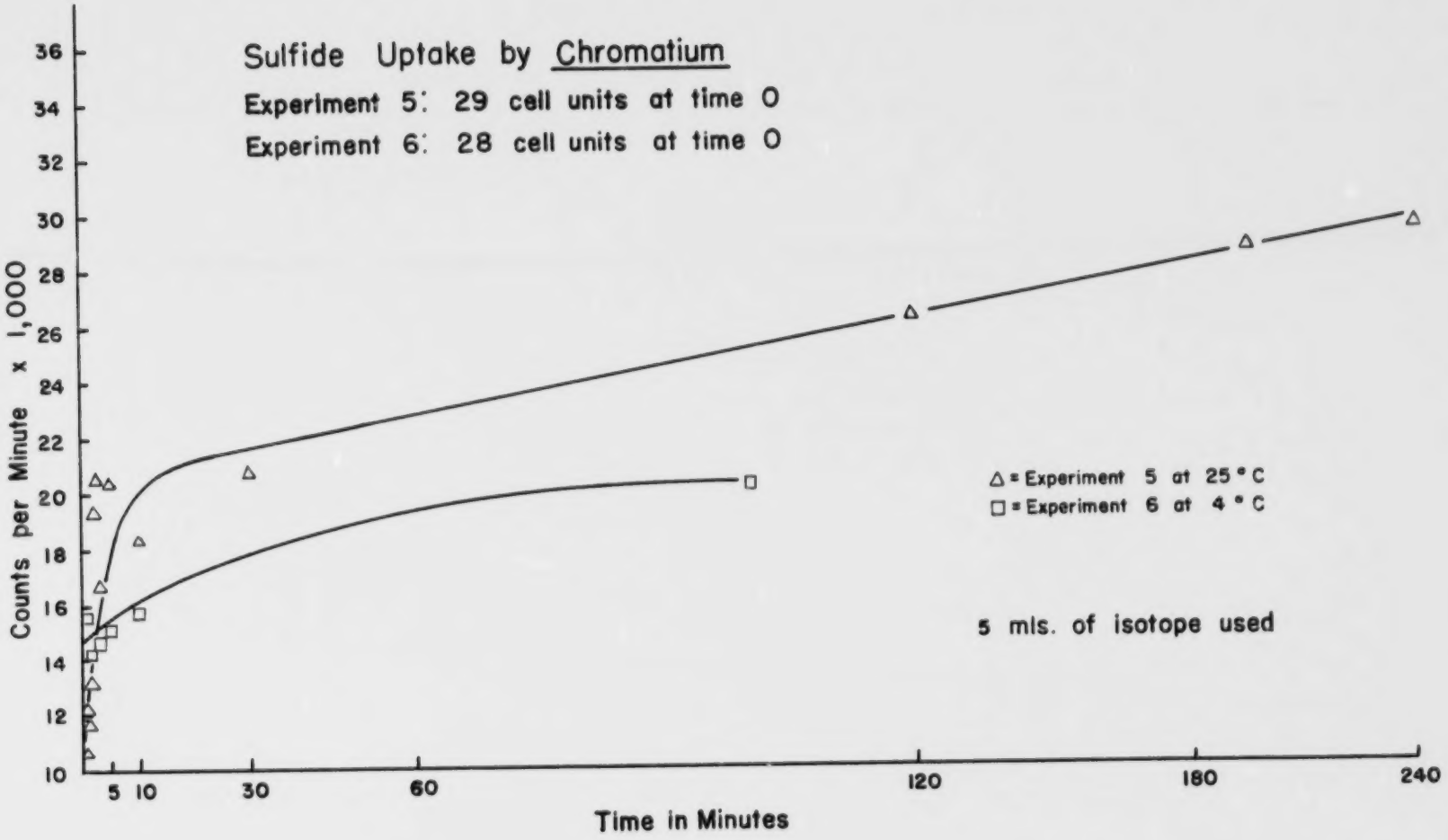
Sulfide Uptake by Chromatium
Experiment 5: 29 cell units at time 0
Experiment 6: 28 cell units at time 0
Counts per Minute x 1,000
36
34
32
30
28
26
24
22
20
18
16
14
12
10
5 10 30 60 120 180 240
Time in Minutes
△ = Experiment 5 at 25 °C
□ = Experiment 6 at 4 °C
5 mls. of isotope used

Fig. 6

against a sulfide concentration gradient. That is, cells with an active transport will assimilate more sulfide into the cell, even though the sulfide content in the cell is at a greater concentration than that outside the cell. If a simple diffusion process were taking place, this would not occur.

Figures 5 and 6 indicate that the sulfide uptake rate was lower when the experiments were operated at $4^{o}C$ than when they were operated at $25^{o}C$. This indicates that active uptake (an enzymatic process) was reduced as a result of the colder temperature.

From these Figures, it can be seen that the use of 5 mls. of isotope had little effect on the rate of uptake, as compared to results found using 2 mls. of isotope. Also, the region of rapid uptake was not extended.

With an active process for accumulation of sulfide in this organism, it has greater significance in removal of sulfide from a lagoon.

Another purple sulfur bacterium, a Thiopedia sp., occurred in the lagoon from which the Chromatium sp. was isolated. Preliminary plans included using this organism in this study. However, growth of the Thiopedia sp. in liquid media was not established in time to include it in the study.

Thiopedia sp. is a much larger organism, and from investigations of the lagoon, it appeared to be more important as a "biological deodorizer" than the Chromatium sp. used. At the time when concentration of the Thiopedia sp. was greatest in the laggons, approximately 10^{9} cells per ml., the concentration of the Chromatium sp. dropped from10^{8} cells per ml. to 10^{7} cells per ml. At this time the sulfide concentration also dropped, possible indicating that the Thiopedia sp. had a greater ability to use sulfide than the Chromatium sp., thus causing the reduction in cell numbers.

During the course of this investigation, it was noted that wide variances occurred in some sample counting rates. This was assumed to be due to the method of sampling. Further investigation revealed small particles of rubber from the vacuum cap on the upper surfaces of the millipore filters. Some of the rubber particles were removed from the filters and counted in the gas-flow proportional counter; these particles indicated high rates of activity. Thus, it was indicated that the rubber particles, if not totally responsible, at least contributed considerably to the higher count rates. Other factors that may have contributed to the variations in sample count rates include the method of sampling and the necessity of haste in the first two minutes of the experiment.

The filtrate samples were taken, expecting uptake by the cells to reduce the amount of sulfide in the filtrate. But, as can be seen from the tables in the Appendix, there was no definite trend in the activity of the filtrate.

This might be due to a number of reasons, such as: (1) adsorption of the radioactive sulfide on the surfaces of the porous glass filter base, or (2) formation of sulfate from surfate from sulfide by the organism, and the return of the sulfate to the medium.

Total samples of the medium and cells (run 5) indicate that the detectable cellular activity during the first 10 minutes represented approximately 7 per cent or less of the total detectable activity. Thus, a significant portion of the activity was in the filtrate. This indicates that it would be difficult to detect a reduction in filtrate activity due to cellular uptake, especially with interference from those errors inherent in sampling and adsorption, etc.

It must also be realized that during the course of this investigation there was a gradual decay of the radioactivity in the isotope stock solution. Thus, as the decay product of S^{35} is Cl^{35} (a stable element), less sulfide was available for the organism per ml. of isotope used. But, as indicated by the amount of activity in the filtrate, this should have had little effect on these results.

Conclusions

Several major conclusions may be drawn from the results of this investigation.

1. It may be concluded from this investigation that there is uptake of sulfide by Chromatium sp.

2. It is feasible to study the metabolism of the purple bacteria, using radioactive sulfide as a tracer material and employing the system and methods used in this study.

3. It appears that the uptake of sulfide in Chromatium sp. was enzymatic in nature.

4. In this investigation two mls. of the radioactive sodium sulfide solution, or 0.09 um of sulfide isotope, were sufficient for detection of sulfide uptake.

5. An increase in the amount of sulfide available had little effect on the rate of sulfide uptake, not the period of time over which rapid uptake occurred.

C. GROWTH

Growth of *Thiopedia* and *Chromatium* sp. on Various Substrates

It has been shown by previous investigators (1), (7), (13), (20) that the purple sulfur bacteria are important in anaerobic lagoons as "biological deodorizers." Because of their importance in this respect, another question arises. Are purple sulfur bacteria of benefit in anaerobic lagoons in capacity other than "biological deodorizers"? It was the purpose of this study to partially answer this question by defining three of the physiological aspects of these organisms. First, to determine the ability of *Thiopedia* and *Chromatium* organisms to utilize various organic substrates. Second, to determine the rate of utilization of three of those substrates which will support growth. Third, to determine whether or not there is a preferential utilization of a reduced sulfur compound over an organic compound.

Materials and Methods

The organisms *Thiopedia* sp. and *Chromatium* sp. used in this study were isolated from an anaerobic lagoon which receives liquid waste from an experimental rat farm south of Pullman, Washington. They were designated *Thiopedia* T1 and *Chromatium* L and are shown in Figures 7 and 8.

Pure cultures of these organisms were grown under anaerobic conditions on media specific for the family Thiorhodaceae. Light for photosynthetic activity was supplied by a 150-watt tungsten filament at a distance of 25 cm. from the growth vessels. Growth required 3 to 4 days at room temperature (22° to 25°C). The equipment and incubation procedures were kept as constant as possible throughout the entire study to prevent any variations due to these factors and also to insure the reproducibility of the results.

Media

The standard media used for the cultivation of *Thiopedia* and *Chromatium* organisms was defined by Larsen (23). The basal salts media contained:
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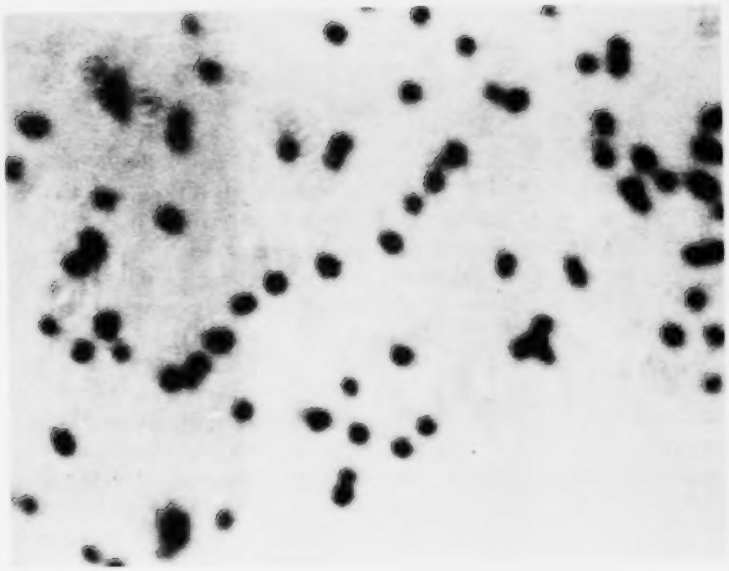

Fig. 7 --Photomicrograph of Thiopedia T1

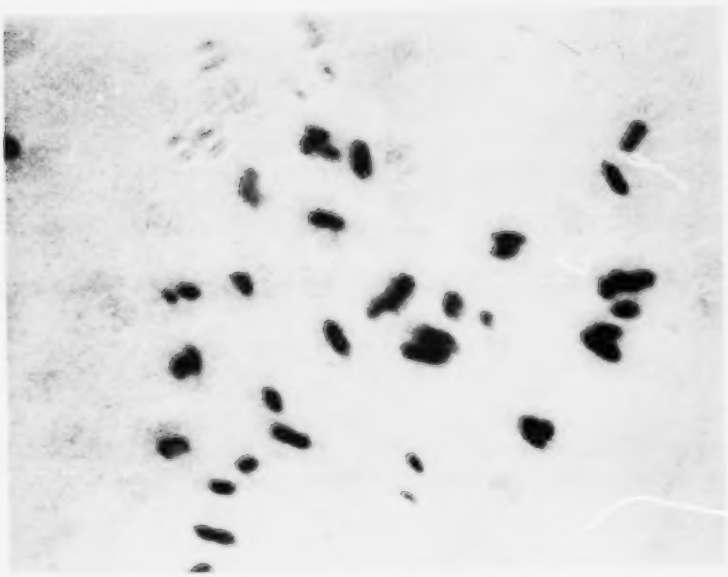

Fig. 8 --Photomicrograph of Chromatium L

Ingredients	g/l	Trace Salts Mixture	mg/l
NH_4Cl	1.0	H_3BO_3	280
KN_2PO_4	1.0	$MnSO_4 \cdot 9H_2O$	210
$MgCl_2 \cdot 6H_2O$	0.5	$CuSO_4 \cdot 5H$	20
$CaCl_2$	0.04	$NaMoO_4 \cdot 2H_2O$	75
Trace Salts Mixture	2.0 mls.	$CoSO_4 \cdot 7H_2O$	45
		$Zn\ SO_4 \cdot 7H_2O$	25
		Versene	100
		Distilled Water	100 mls.

Ferric Citrate (0.7 g/l) was added to the basal salts media immediately prior to sterilization by autoclaving.

To the cooled media, the following supplements were added as sterile solutions (g/l): $Na_2S_2O_3 \cdot 5H_2O$, 3; $NaHCO_3$, 5; and $Na_2S \cdot 9H_2O$, 0.1. Immediately before addition of the sterile $NaHCO_3$, oxygen free carbon dioxide was bubbled through it for 10 minutes to displace any dissolved oxygen.

Solid media used for stab cultures to facilitate storage of the organisms was identical to the standard liquid media with the exception that 1.0 per cent purified agar was added as a solidifying agent.

Growth of Cultures

Initially, the isolates were grown up in stab cultures using the solid media as described previously. These cultures were incubated from 5 to 10 days until a dense growth was observed. They were then stored at 4°C. Before each phase of the study, organisms from these stock cultures were aseptically transferred to liquid media and were incubated until a dense growth could be observed visually. This step was primarily a precaution to prevent the possibility of any growth lag the organisms might exhibit when adjusting from solid media to liquid media.

The organisms were grown in 60-ml., pyrex, ground-glass stoppered flasks. These flasks were routinely filled to the neck with liquid media before stoppering to minimize the amount of trapped oxygen.

Phase One -- The first phase of the study was a survey to determine whether or not Thiopedia T1 and Chromatium L. could utilize various organic substrates. The substrates used in this study included propionate, pyruvate, lactate, acetate, formate, fructose, and glucose. Five-ml. aliquots of previously grown liquid cultures were inoculated into standard 60-ml. flasks containing one of the organic test substrates in place of sodium thiosulfate in one case and in place of both sodium thiosulfate and sodium bicarbonate in the second case. After incubation for a period of 8 days, growth was determined by visual observation.

To be certain that there was no carry-over from the initial liquid cultures to the test flasks, a second run was conducted. Five-ml. aliquots were taken from the flasks that showed growth in the first run and were innoculated into identical media for the second run. These flasks were incubated for an 8-day period and again visual observations of growth were taken.

In both runs the initial and final pH were measured to determine whether the hydrogen ion concentration was adequate for satisfactory growth of the organisms. The initial samples for pH determination were taken immediately after inoculation. The samples for final pH determination were taken directly from the flasks immediately after growth observations had been made.

Phase Two -- The second phase of the study was designed to determine the rate of utilization of three of the organic substrates that were capable of supporting growth. These substrates were glucose, pyruvate, and acetate.

Initially, 5-ml. aliquots of previously grown liquid cultures were inoculated into flasks containing one of the organic test substrates in place of sodium thiosulfate. These flasks were incubated for a four-day period. At the end of this time, the organisms were assumed to have reached the logarithmic growth phase, and that there would be no growth lag when they would be transferred to the test flasks. After the four-day incubation period, 5-ml. aliquots were transferred to a series of six flasks each, each series containing one of the organic test substrates and Thiopedia T1 or Chromatium L. These flasks were incubated and sampled at intervals of 0, 12, 24, 36, 60 and 84 hours; e.g. the first flask was sampled 12 hours after inoculation, and so on.

Three samples were taken from each flask. These samples consisted of a 4-ml. aliquot for pH determination; a 4-ml. aliquot for the determination of organic substrate concentrations as an indication of substrate uptake; and a 50-ml. aliquot for the determination of total cellular nitrogen as an indication of cell growth.

Phase Three -- The third phase of the study was to determine if there is a preferential utilization of reduced sulfur compounds over organic

compounds. Except for the use of a mixture of sodium thiosulfate and acetate as the substrate in phase three, it was conducted in an identical manner with phase two.

Four samples were taken from each flask. These samples consisted of 4 mls. for pH determination ; 4 mls. for acetate determination; 4 mls. for thiosulfate determination; and 25 mls. for total cellular nitrogen determination.

Samples Analysis

With the exception of pH determinations, all samples were frozen until analysis could be performed. All pH values were determined immediately after sampling with a Beckman Expanded Scale pH Meter.

Total cellular nitrogen samples were centrifuged, washed, and re-centrifuged immediately after sampling. The cell pellets were then frozen for future analysis. Nitrogen determinations were carried out according to the Kjedahl method as described in "Standard Methods for the Examination of Water and Waste Water" (1960) (37).

The glucostat method (4) was used to determine glucose concentrations. Glucose was oxidized to gluconic acid resulting in the following equation:

$$\text{Glucose} + H_2O + O_2 \xrightarrow[\text{oxidase}]{\text{Glucose}} \text{gluconis acid} + H_2O_2$$

The hydrogen peroxide was then reduced with peroxidase, and the oxygen liberated oxidized the reduced chromogen indicator resulting in the equation:

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{Peroxidase}} H_2O + \text{oxidized Chromogen}$$

The amount of indicator oxidized (colorimetric measurement) is a measure of the glucose present.

Pyruvate determinations were carried out by an enzymatic method described in "Methods of Enzymatic Analysis" (1963) (4). Pyruvate was catalyticly reduced with reduced diphosphopyridine nucleotide (DPNH) resulting in the equation:

$$\text{Pyruvate} + \text{DPNH} \xrightarrow{\text{LDH}} \text{Lactate} + DPN^+$$

The reduced optical density due to oxidation of DPNH is stiochiometrically related to the pyruvate concentration.

Acetate was determined by gas chromatography, using a Beckman, GC-2, hydrogen flame, gas chromatograph (18).

Thiosulfate was estimated by over-titration with iodine and back titrating with thiosulfate using paragon starch as an indicator.

Results and Discussion

Phase One

The results of the survey to determine if Thiopedia T1 and Chromatium L utilize specific organic compounds as sources of both electrons and carbon are presented in Table I. Seven organic compounds were surveyed. These compounds included two sugars (fructose and glucose), five acid salts (acetate, pyruvate, lactate, formate, and propionate).

The results indicate that Thiopedia T1 and Chromatium L are capable of utilizing a variety of monsaccharides and low molecular weight organic acids. It appears that Chromatium organisms are capable of utilizing a wider variety of organic compounds and for this reason may be of more benefit in lagoons than Thiopedia organisms.

The results also indicate that the before most organic materials can be utilized some external source of inorganic carbon must be present. This was true of all organic materials surveyed with the exception of pyruvate. It is apparent, therefore, that many organic materials can act as electron donors in the metabolism of Thiopedia and Chromatium organisms, but only a very few can act as carbon sources. Although these organisms are removing organic material from their environment, they are at the same time synthesizing new organic material from an inorganic carbon source. From the results it cannot be established whether or not the metabolism of organic material results in an increase or decrease in the net amount of organic material present. It is felt, however, that for purposes of organic removal in stabilization lagoons these organisms provide little if any benefit. On the other hand, it appears that Thiopedia and Chromatium organisms are capable of substituting cell material for offensive and undesirable organic acids which tend to impart tastes and odors to the water. For this reason alone Thiopedia and Chromatium organisms could be considered beneficial in stabilization lagoons.

Phase Two

Many parameters have a substantial effect on the rate of growth of photosynthetic microorganisms. Some of the more important ones include light intensity and wave length, temperature, pH, initial substrate concentrations, initial microorganisms concentrations, and initial concentration of various nutrients and trace compounds. Steps were taken at the onset of the study to keep the above-mentioned parameters constant within practical limits. It was found, however, that to hold the pH and the initial microorganisms concentrations constant would require far too elaborate equipment

TABLE I

Visual Observations of Growth of Chromatium L and Thiopedia T1 on Various Organic Substrates

Substrate	Thiopedia (+)*			Thiopedia (-)**			Chromatium (+)			Chromatium (-)		
	Growth	pH		Growth	pH		Growth	pH		Growth	pH	
		Initial	Final		Initial	Final		Initial	Final		Initial	Final
Fructose	2+	7.6	7.5	0	7.2	7.4	2	7.5	7.8	0	7.3	7.4
Glucose	3	7.6	7.6	0	7.3	7.4	2	7.6	7.9	0	7.4	7.4
Acetate	3	7.7	7.8	0	7.2	7.6	4	7.7	7.6	0	7.1	7.2
Pyruvate	4	7.7	8.1	3	7.4	7.7	4	7.7	8.1	3	7.2	7.8
Lactate	0	7.6	8.4	0	7.1	7.1	4	7.4	7.6	0	7.0	7.0
Formate	0	7.5	8.6	0	7.2	7.2	1	7.5	7.7	0	7.3	7.5
Propionate	3	7.4	8.2	0	7.3	7.0	3	7.6	7.9	0	7.3	7.3

* + indicates the presence of bicarbonate.

** - indicates the absence of bicarbonate.

† Growth was recorded on a five-unit scale with zero indicating no growth and four indicating maximum growth.

and procedures for a study of this type. It is suggested, therefore, that any reference to the following data take into consideration the recorded variations in these two parameters. As can be seen in Tables 2 and 4, there was a maximum variation in pH values over the entire study from a low of 7.5 to a high of 9.1. Although this variation may result in growth changes, it has been shown that this range is adequate for satisfactory growth of Thiopedia and Chromatium organisms (15). Any effects due to variations in initial microorganism concentrations should also be minimized by the steps taken to provide initial growth rates in the logarithmic growth range.

The results of this phase of study are presented in graphical form in Figures 9, 10, 11, and 12. Figures 9 and 11 are semilog plots of substrate uptake by Thiopedia T1 and Chromatium L, respectively. The results are also presented in tabular form in Tables 2 and 3.

Samples were taken at intervals from 0 to 84 hours. The results of nitrogen samples at 0 time for Thiopedia T1 were lost and, therefore, could not be presented in the data.

Thiopedia -- As shown in Figure 9, Thiopedia T1 exhibited logarithmic rates of substrate utilization in the initial 24 hours, after which the rates decreased rapidly to approximately zero at or before 84 hours. These logarithmic rates can be described by the general equation:

$$-\frac{dY}{dt} = k_1 Y$$

where: Y = units on the ordinate remaining after time t
t = time in hours
k_1 = rate constant in /hr.

This equation was previously proposed by Streeter and Phelps for the rate of aerobic, biochemical oxidation of organic matter (40). Since its proposal, the equation has become widely accepted as a basic tool for the determination of rate of B.O.D. exertion in natural receiving streams. The rate constant "k_1" has been used to describe the rate at which a specific organic material or combination of organic materials will exert B.O.D. Since this is a general equation for a straight line on a semilogarithmic plot, the rate constant "k_1" will be used to describe both the rates of logarithmic substrate utilization and the rates of logarithmic growth where they exist in this study. The rate constants for substrate utilization exhibited by Thiopedia T1 were 0.018, 0.015, and 0.007 /hr. for acetate, pyruvate, and glucose, respectively. This large variation in the rate of utilization, especially between glucose and acetate, indicates that the enzymes responsible for decomposition of various substrates attack acetate more rapidly than glucose.

The growth curves in Figure 10 indicate that logarithmic growth occurred in the initial 24 hours which has good correlation with logarithmic rates of substrate utilization also occurring in the initial 24 hours.

SUBSTRATE UPTAKE BY THIOPEDIA T1

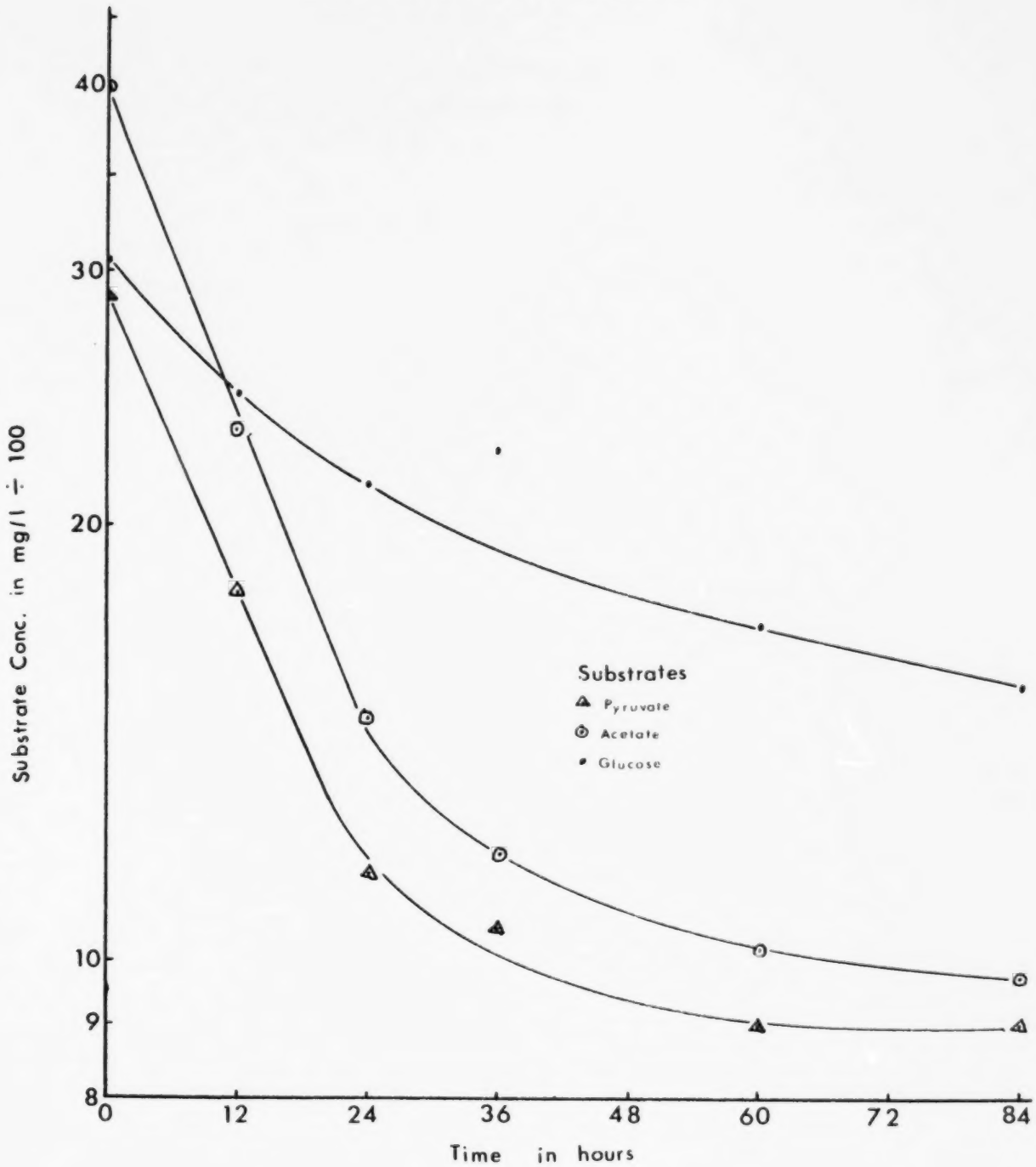

Fig. 9

CELL NITROGEN OF THIOPEDIA T1.
ON VARIOUS SUBSTRATES

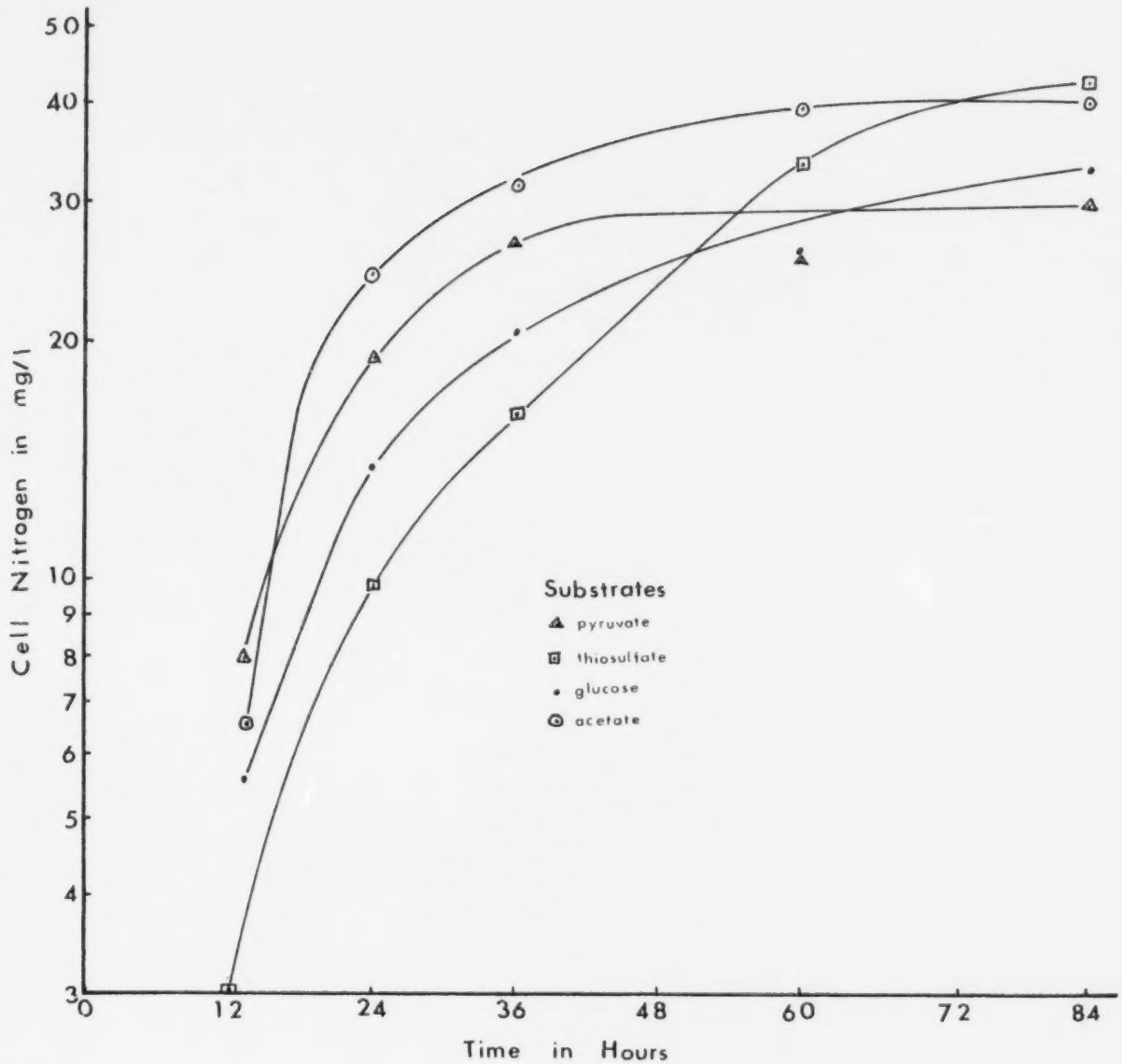

Fig. 10

SUBSTRATE UPTAKE BY CHROMATIUM L

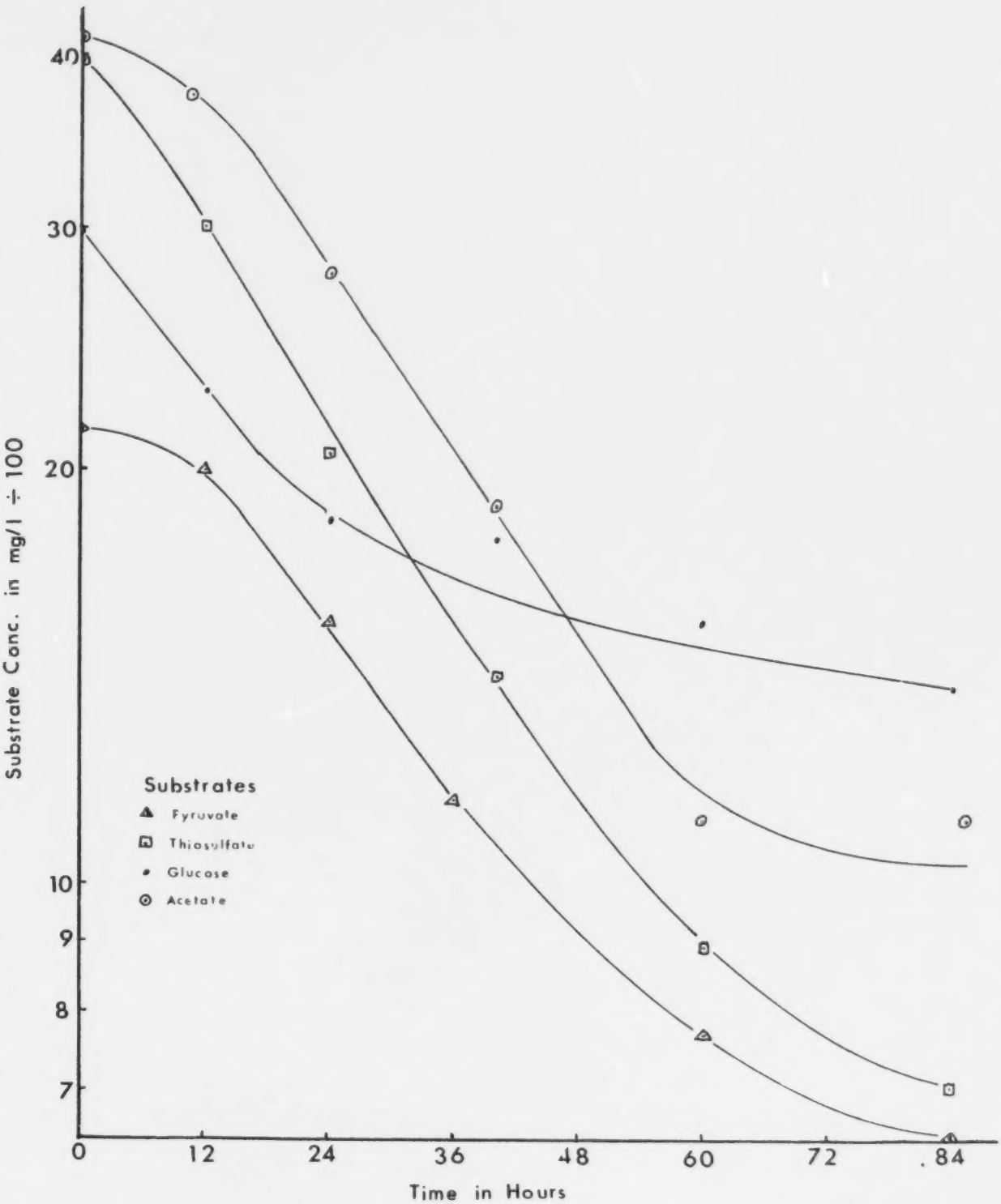

Fig. 11

CELL NITROGEN OF CHROMATIUM L.
ON VARIOUS SUBSTRATES

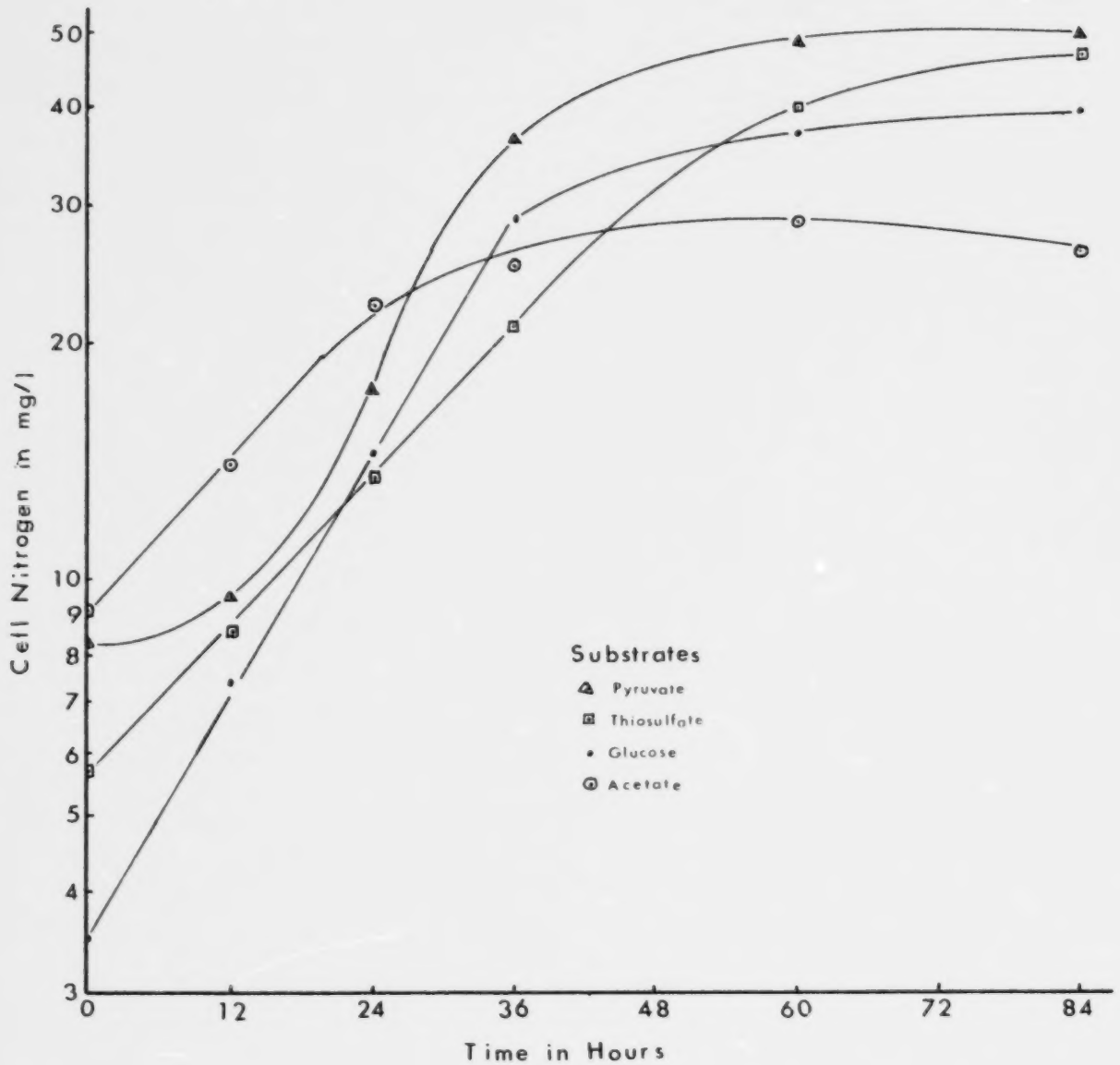

Fig. 12

Rate constants for growth were 0.109, 0.054, and 0.003 /hr. on acetate pyruvate, and glucose, respectively. A comparison of growth rates with utilization rates shows that an increase in utilization rate generally results in a corresponding increase in growth rate. This is not to say, however, that an increase in the total amount of substrate utilized results in an increase in total growth. The ratios of total growth to the total amount of substrate utilized indicate that more cell material was synthesized per unit of glucose utilized than on any of the other substrates. It is evident, therefore, that although glucose is utilized at a relatively low rate, Thiopedia T1 is capable of using it more efficiently or more completely in the synthesis of new cell material. A valid evaluation of possible benefits that may occur from high to low growth per unit of substrate utilized cannot be made with the data available. Such an evaluation would require an analysis of the decomposition products of the various substrates.

Thiosulfate as a substrate in this phase of the study was used primarily as a control. It should be mentioned that growth was generally slower on thiosulfate than on organic materials. Growth continued at a relatively uniform rate for a longer period of time, however, resulting in a larger total growth on thiosulfate than on the organic substrates.

Chromatium -- As can be seen from Figure 11, Chromatium L utilized the various substrates at logarithmic rates from 12 to 60 hours with the exception of glucose. The rate of glucose utilization was logarithmic during the first 24 hours only. Between 60 and 84 hours the rates decreased very rapidly to approximately zero. There seemed to be a lag in the rate of utilization of acetate and pyruvate in the initial 12 hours. This indicates that the steps taken to provide initial conditions of logarithmic growth were inadequate. This first 12-hour period was neglected, therefore, when rate calculations were made. Rate constants for substrate utilization were 0.012, 0.011, 0.009 /hr. for thiosulfate, acetate, pyruvate, and glucose, respectively.

Contrary to the results obtained for Thiopedia T1, changes in the rate at which Chromatium L utilized the various substrates did not result in immediate changes in cell growth (Fig. 12). Decreases in rate of utilization lagged the corresponding decreases in growth by 24 and 36 hours for acetate and pyruvate, respectively. The reason for this lag is not immediately apparent. Possibly the enzymes responsible for the decomposition of these substrates continue their activity for some time after a limiting growth factor has been exhausted. On the other hand, growth continued on glucose at a logarithmic rate for 12 hours after the rate of glucose utilization began to decrease. From this data, it seems reasonable to assume that the organisms are utilizing by-products of glucose decomposition.

The rate constants for growth of Chromatium L were 0.034, 0.025, 0.016, and 0.016 /hr. on pyruvate, glucose, acetate and thiosulfate, respectively. In comparing the relative rates of growth with the relative

rates of substrate utilization an inverse relationship was observed. For example, Chromatium L grows much faster on pyruvate than on the other substrates, but the rate of pyruvate utilization is slower than the other substrates. Although this phenomena cannot be explained with the results from this study, it presents an interesting area for future investigation.

As previously described for Thiopedia T1, a large variation in cell growth per unit of substrate utilized was observed for cultures of Chromatium L. Again, the significance of this phenomena cannot be explained without additional study.

A comparison of the results for Thiopedia T1 and Chromatium L indicates two basic differences: First, Thiopedia T1 has, in general, higher rates of substrate utilization and growth over relatively short periods of time in comparison to Chromatium L; and second, Thiopedia T1 seems to have a direct relationship between rates of substrate utilization and rates of growth, whereas Chromatium L seems to have an inverse relationship.

Phase Three

The results of the study to determine whether or not there is a preferential utilization of reduced sulfur compounds (thiosulfate) to organic compounds (acetate) are presented graphically in Figures 13 and 14. Figure 13 is a linear plot of substrate uptake by Thiopedia T1 and Chromatium L. Figure 14 is a semilog plot of cell nitrogen (cell growth) for both organisms. The results are also presented in tabular form in Table 4.

As can be seen from Figure 13, thiosulfate was utilized by both Thiopedia T1 and Chromatium L at logarithmic rates during the initial 24 hours. On the other hand, acetate was not utilized at its maximum rate until 12 hours after inoculation. Since both organisms were in the logarithmic growth phase at time zero (Fig. 14), the lag in acetate utilization cannot be attributed to an initial growth lag. It seems reasonable to assume therefore, that both organisms exhibited a slight preference for thiosulfate during the initial 12 hours.

Although this study was limited, it seems to indicate that purple sulfur bacteria exhibit a slight preference for thiosulfate, a reduced sulfur compound, over acetate, an organic compound. A much more extensive and closely controlled study will be necessary before such a preference can be definitely established. It should be mentioned, however, that such a preference for reduced sulfur compounds would indicate that a primary role of the purple sulfur bacteria in lagoons is odor removal.

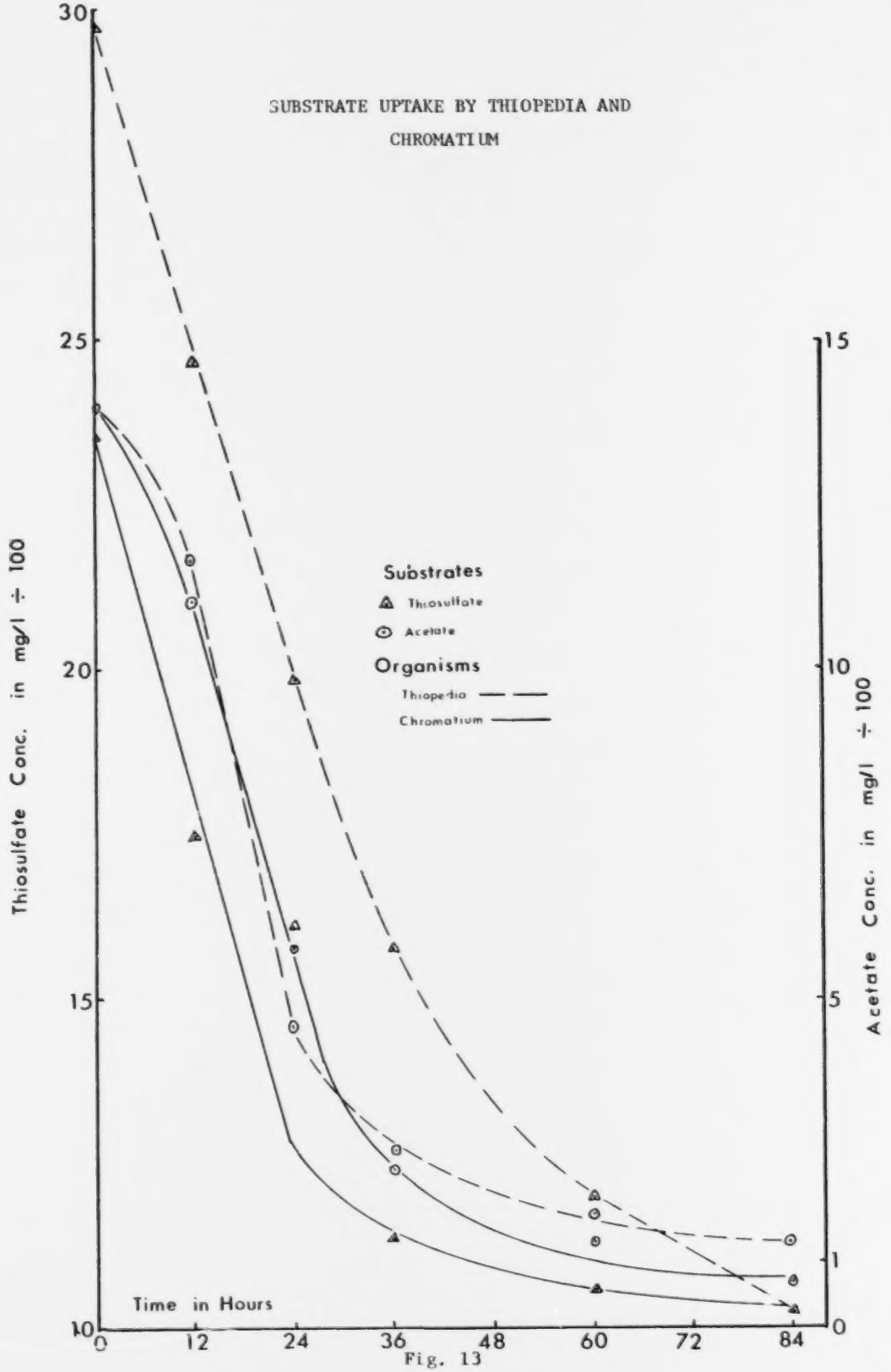
SUBSTRATE UPTAKE BY THIOPEDIA AND CHROMATIUM
Thiosulfate Conc. in mg/l ÷ 100
Acetate Conc. in mg/l ÷ 100
Time in Hours
Substrates
Thiosulfate
Acetate
Organisms
Thiopedia
Chromatium
30
25
20
15
10
15
10
5
1
0
0
12
24
36
48
60
72
84

Fig. 13

CELL NITROGEN FOR

CHROMATIUM L AND THIOPEDIA T1

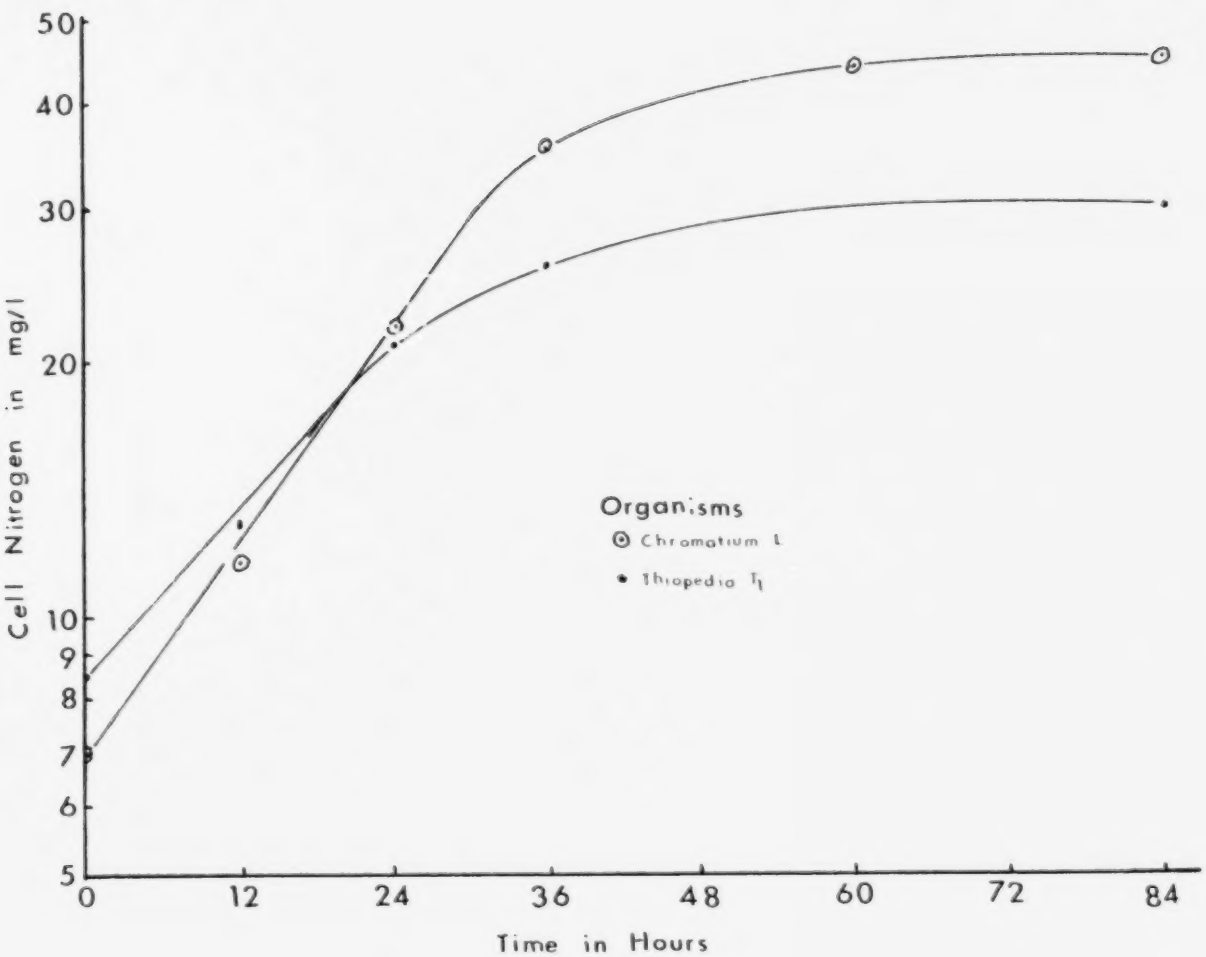

Fig. 14

Conclusions:

Several conclusions can be drawn from the results of this study.

1. It may be concluded that fructose, glucose, acetate, pyruvate, and propionate are capable of supplying electrons in the metabolisms of Thiopedia Tl.

2. Fructose, glucose, acetate, pyruvate, lactate, formate, and propionate are capable of supplying electrons in the metabolism of Chromatium L.

3. With the exception of pyruvate, the organic compounds surveyed are not capable of supplying carbon for synthesis of new cell material.

4. Thiopedia Tl has rate constants for substrate utilization of 0.018, 0.016, and 0.007 /hr. for pyruvate, glucose, acetate, and thiosulfate, respectively.

5. Thiopedia Tl appears to be capable of utilizing and producing new cell material on organic material at significantly higher rates than Chromatium L.

6. Both organisms seem to exhibit a slight preference for thiosulfate over acetate.

D. The Effect of Certain Ecological Factors on the Growth of Thiorhodaceae

To date, little work has been conducted on the environmental needs of the purple sulfur bacteria. It was the purpose of this study to attempt to determine some of the ecological effects of temperature, light intensity, dissolved oxygen, and reduced sulfur compounds upon the growth of these bacteria. Attempts were also made to determine optima for the four ecological factors as well as the extremes tolerated.

Growth of Cultures

Standard methods of isolation, designation, and culture have been previously described in sections B and C.

All organisms were grown in 60 ml., pyrex, ground-glass stoppered bottles. These bottles were filled to the neck with liquid media before stoppering to minimize the amount of trapped oxygen. These stock cultures were stored at 11°C. Periodically, new stock cultures were made to insure the use of viable organisms that were 4 to 5 days old.

Phase One

The first phase of this investigation was to determine the optium concentration of reduced sulfur compounds for the growth of Thiopedia and Chromatium. Before the actual test runs, sample runs were made to determine which reduced sulfur compounds in the medium should be varied. It was decided that the $Na_2S_2O_3$ should be used as the variant since the Na_2S alone did not result in any appreciable growth.

The media used was identical in all respects to the standard media with the exception of the concentration of $Na_2S_2O_3$. The standard media was prepared and then equal volumes were put into separate flasks before autoclaving. After autoclaving, the media was cooled and the remaining constituents were added aseptically. The appropriate volume of $Na_2S_2O_3$ was added to give the desired concentration.

The cultures were incubated in a New Brunswick Incubator Shaker Model G26 for a period of five days at 26°C 19 cm. from a 100-watt tungsten filament lamp. The light intensity was 160 foot-candles. The initial and final pH of the medium were taken on all cultures to determine if the hydrogen ion concentration was satisfactory for growth and whether there was any significant change in hydrogen ion concentration during the growth period.

Phase Two

This phase was concerned with the determination of temperature effects on the growth of the Thiopedia and Chromatium organisms. The standard media containing 3.0 mg/l 10% $Na_2S_2O_3$ was used for all temperature series. The series were run in the controlled environment of the previously described incubator with a constant light intensity of 160 foot-candles.

With the temperature series, a control series using identical media and inoculum was set up outside the incubator at a temperature of approximately 22°C and 140 foot-candles of light. This was done at all temperatures so that a correlation between different temperature series could be made. Also, this would indicate whether a lack of growth at some particular temperature was due solely to that temperature or some other environmental condition such as a variation in media or toxic conditions.

After a four day incubation period, each temperature series was harvested and stored along with the control cultures. Temperatures selected for this phase were 15°C, 25°C, 30°C and 45°C. The initial and final pH were also taken to determine the initial hydrogen ion concentration and the change that took place.

Phase Three

Phase three was the determination of the effect of light intensity on growth of the organisms.

The standard media with 3.0 mg/l, 10^5 $Na_2S_2O_3$ was used throughout. Light intensity ranges were obtained by varying the distance from the culture bottles to the light source -- a 150-watt tungsten filament lamp. The temperature throughout the phase was nearly constant at 26°C.

In this phase, as in Phase Two, a control series was run so that correlation between varying light series could be made. These control cultures were grown at a temperature of 22°C and a light intensity of 140 foot-candles. Since the hydrogen ion concentration had been consistently favorable in the preceeding phases, the pH was not taken in the light series.

Phase Four

The fourth and final step was to determine, if possible, the range of oxygen concentration which the Thiopedia and Chromatium would tolerate.

The standard media was used containing 3.0 mg/l, $Na_2S_2O_3$. A control series containing Na_2S was set up so a comparison between an absolute anaerobic environment and one which was aerobic could be made. Since a DO probe could not be relied upon, the Winkler method using 20 ml. samples, was used to determine the initial oxygen concentrations. The only measure of oxygen concentration obtainable was the initial determination because once the cultures were started there was no longer any control over oxygen concentration.

Separate flasks containing equal volumes of media were autoclaved, and after cooling the correct quantity of $NaHCO_3$ was added to each. In those flasks providing aerobic conditions, no Na_2S was added. Since the $Na_2S_2O_3$ interfered with the Winkler method, this nutrient was added after the initial oxygen concentration was determined.

Compressed air was bubbled through the media after being passed through a sterile bacteriological filter. The media was then checked for dissolved oxygen content, and this was used as a culture substrate. The cultures were incubated at 30°C at a light intensity of 160 foot-candles in the New Brunswick incubator.

pH

The initial pH of the media was taken in all early phases of the investigation. The pH of the individual cultures was also taken after the growth period. In all cases, the pH levels ranged from 6.3 to 8.1.

Sampling of Cultures

After the appropriate growth period, culture samples were taken from the growth vessels to be used in determining final growth results. Thirty mls. of culture were taken from each bottle and centrifuged for 10 min. at 8,000 rpms. The cells were then washed with 40 mls. of deionized, distilled water and again centrifuged as before. This was done in an attempt to reduce the quanitity of ammonia nitrogen present in the remaining substrate to a minimum level. After one washing, the supernatant was discarded and the remaining cell buttons were transferred to capped test tubes for storage in a deep freezer.

Growth Determinations

Growth determinations were made on the stored cell buttons by the Kjeldahl organic nitrogen method presented in "Standard Methods for the Examination of Water and Waste Water" (37).

RESULTS AND DISCUSSION

Phase One

The results of this portion of the investigation are shown in Table II and Figure 15. The data shown for $Na_2S_2O_3$ concentrations from 1,000 ppm to 6,000 ppm are the average of three series of runs. The data for concentrations are the average of two series of runs.

There is a slight indication of growth at zero concentration of $Na_2S_2O_3$ for both organisms. A portion of this growth is the result of the population of the organisms in the inoculum. The remainder may be attributed to a minimum of growth in this substrate containing only Na_2S.

Figure 15 shows an increase in growth of Thiopedia for all concentrations used. The curve shows a near constant increase in growth with each increase in $Na_2S_2O_3$ at the lower concentrations, but at $Na_2S_2O_3$ concentrations reach higher levels further increases in growth are reduced and maximum growth is approached at a $Na_2S_2O_3$ concentration of 10,000 ppm. Investigation of higher concentrations may indicate a maximum tolerable limit. However, this study was not carried any further, since it was concluded that concentrations this high are not likely to be found in waste effluents.

The results with Chromatium, on the other hand, are quite different. The growth rate is maximum from $Na_2S_2O_3$ concentrations of zero to approximately 4,000 ppm. Maximum growth is reached at an optimum value of 6,000 ppm and any further increase in $Na_2S_2O_3$ concentration results in a decrease in growth. The difference in the reaction of the two organisms to the same substrate is not at all unreasonable. It is quite possible that the

Table II

GROWTH OF THIOPEDIA AND CHROMATIUM
IN SUBSTRATE OF VARYING $Na_2S_2O_3$ CONCENTRATION

10% $Na_2S_2O_3$ ppm	THIOPEDIA T-1			CHROMATIUM L		
	pH		Cell N mg/l	pH		Cell N mg/l
	Initial	Final		Initial	Final	
0	7.9	7.5	8	7.9	7.4	8
500	7.9	7.4	21	7.9	7.3	13
1000	7.8	7.2	24	7.8	7.2	22
2000	7.7	7.1	43	7.7	7.1	41
3000	7.7	7.0	55	7.7	6.8	61
4000	7.7	6.6	68	7.7	6.6	76
5000	7.7	6.5	77	7.7	6.3	91
6000	7.9	6.3	85	7.9	6.7	97
8000	8.0	7.1	83	8.0	7.3	72
10,000	7.9	7.0	95	7.9	7.2	49

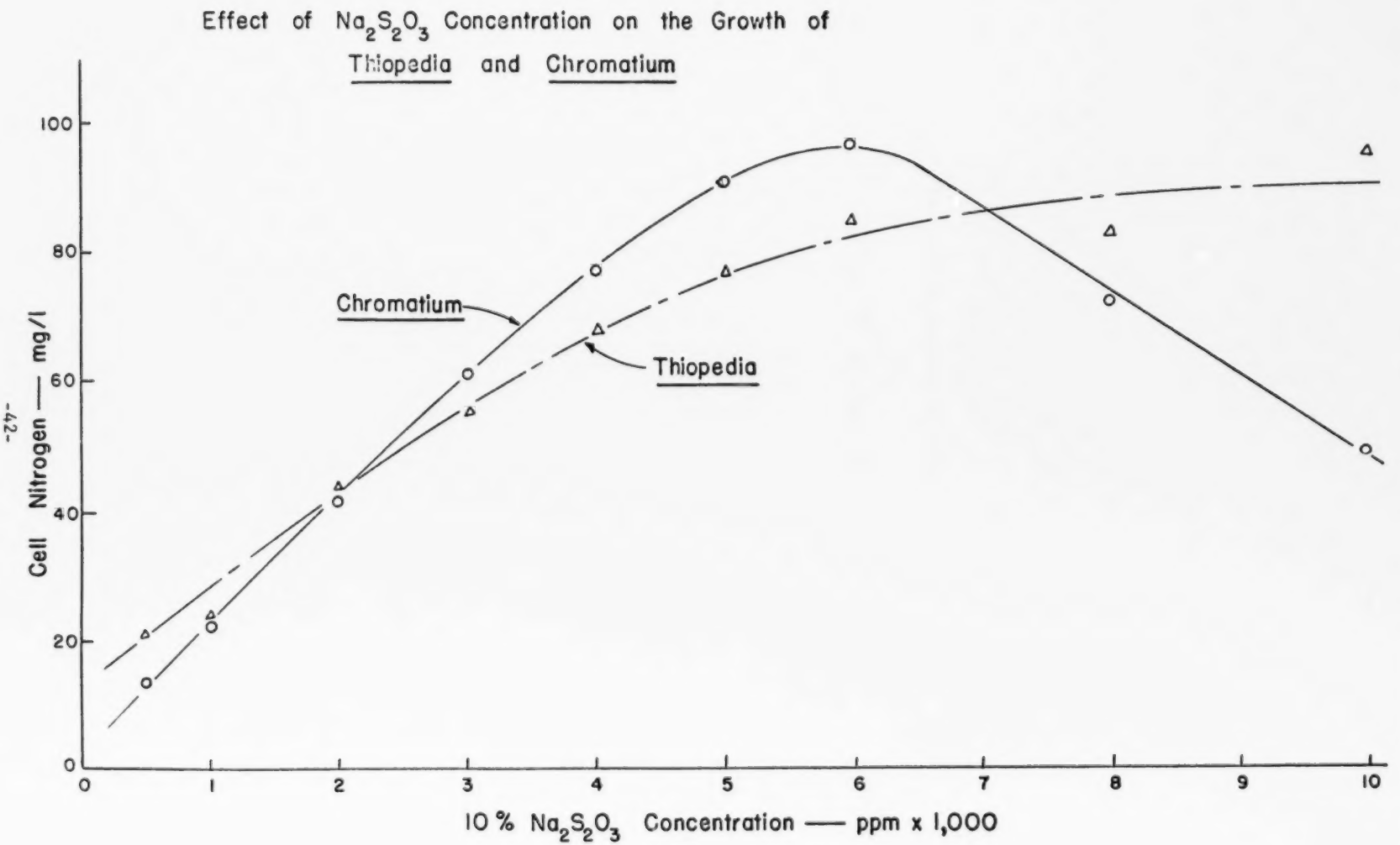
Effect of $Na_2S_2O_3$ Concentration on the Growth of
Thiopedia and Chromatium
Cell Nitrogen — mg/l
100
80
60
40
20
0
Chromatium
Thiopedia
0
1
2
3
4
5
6
7
8
9
10
10 % $Na_2S_2O_3$ Concentration — ppm x 1,000

Fig. 15

two organisms have dissimilar metabolic reactions to concentrations of $Na_2S_2O_3$ (42, pp. 229-233, 235-236).

The results indicate that any normal concentrations of $Na_2S_2O_3$ will support growth quite adequately and that there is a straight-line relationship between growth and the lower concentrations of $Na_2S_2O_3$. In general, only a total lack of this reduced sulfur compound resulted in insufficient growth. The results show that the supply of reduced sulfur compounds does limit the growth of Thiopedia at all tested concentrations, and up to 6,000 ppm for Chromatium.

Phase Two

As would be expected, temperature has a marked effect on the growth of both Thiopedia and Chromatium. Optimum temperature for growth is near 30°C. Growth is at maximum and fairly constant from a temperature of 25°C to 35°C with an optimum of 30°C (indicated in Figure 16) for both organisms. Growth is moderately supported at temperatures of 20°C and 40°C, but outside this range there is a sharp decline in growth, and at 15°C and 45°C growth is very slight. Some of the growth indicated at these temperatures is due to the cell population in the inoculum. However, there is evidence of substantial growth of certain species of these organisms at temperatures well below those shown here to be necessary for growth (33, pp. 147-149).

The results of this phase provide an explanation for the absence of a population of these organisms from local ponds in the winter time when water temperatures are quite low.

Effect of Temperature on the Growth of Thiopedia and Chromatium

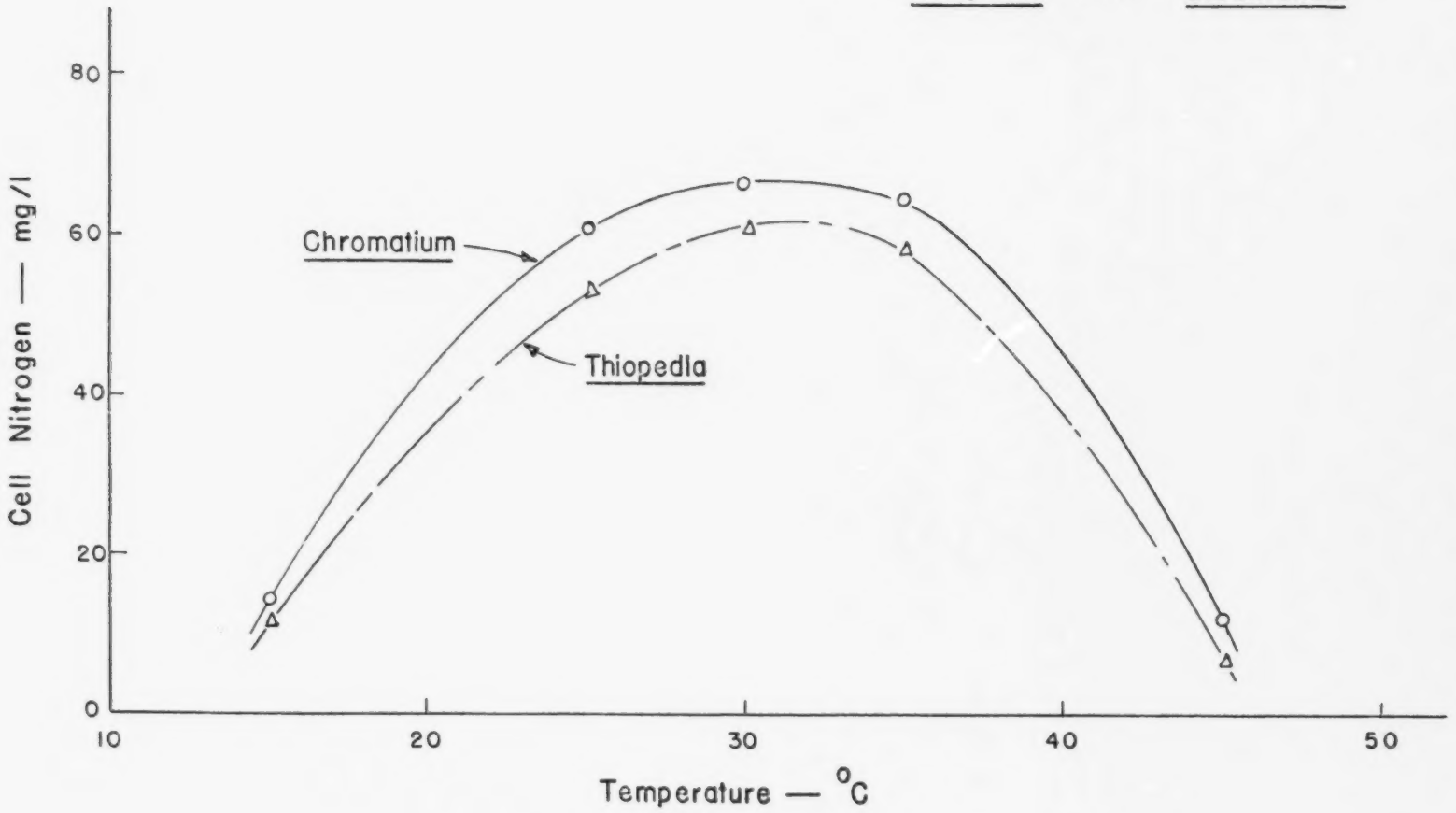

Fig. 16

Table III

EFFECTS OF GROWTH OF THIOPEDIA AND CHROMATIUM
UNDER CONDITIONS OF VARYING TEMPERATURE

TEMPERATURE	THIOPEDIA			CHROMATIUM		
	Cell N mg/l		Cell N Proportioned to Control Value of 61 mg/l	Cell N mg/l		Cell N Proportioned to Control Value of 64 mg/l
	Sample	Control		Sample	Control	
15°C	11	58	12	13	60	14
25°C	55	--	55	61	--	61
30°C	56	57	60	57	55	66
35°C	56	59	58	54	54	64
45°C	7	61	7	12	64	12

Phase Three

The results of this phase are quite interesting. If a plot of light intensity vs. cell nitrogen is made using the data from Table VII and not considering the control cultures, a curve as in Figure 17 results. This shows the effects of very low light intensities on growth, and then the increase in leveling off of growth as the light intensity is increased. These results follow those of Shaposhuikov et al. (35).

Effect of Light Intensity on the Growth of
Thiopedia and Chromatium
(not adjusted for Control results)

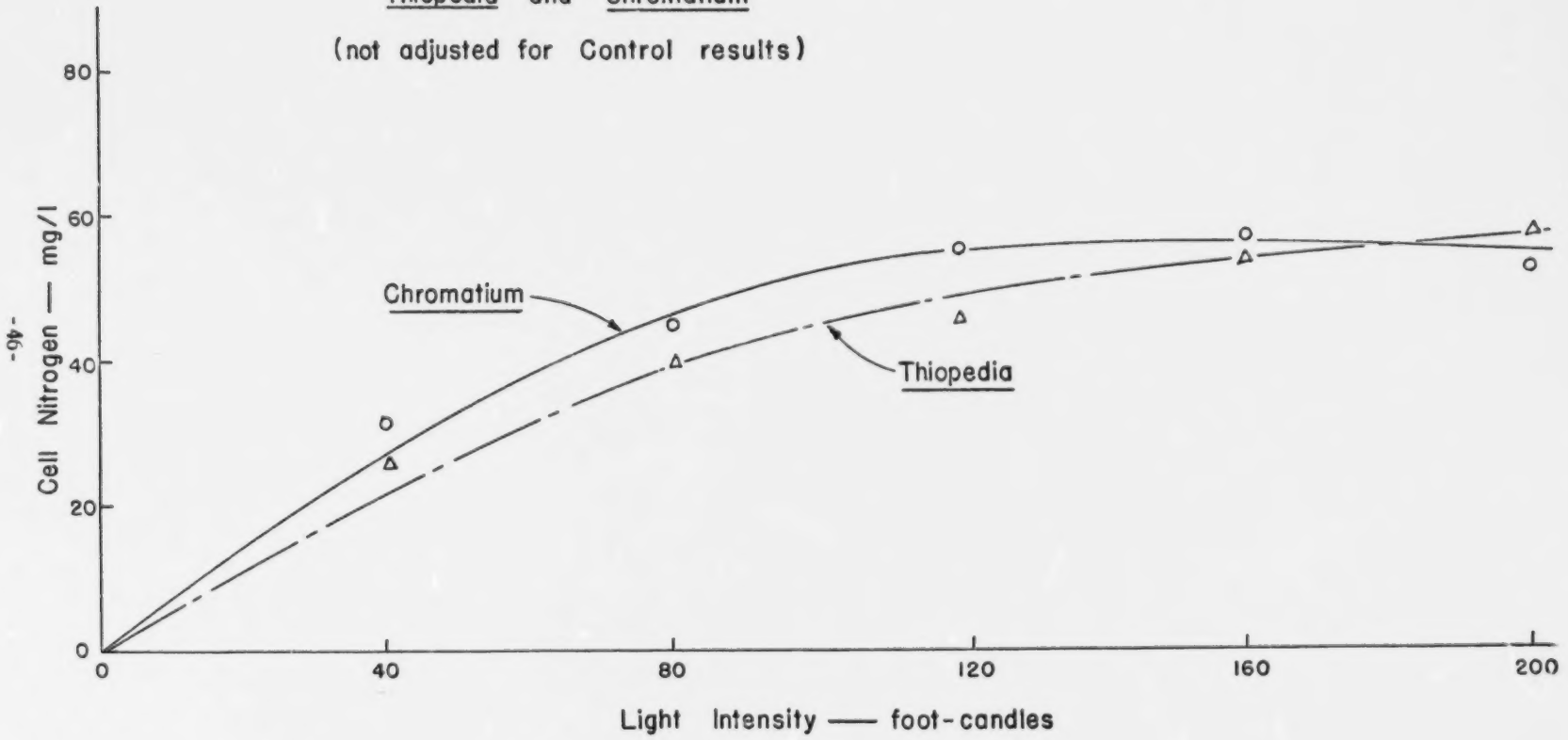

Fig. 17

Table IV

EFFECTS OF LIGHT INTENSITY ON THE GROWTH
OF THIOPEDIA AND CHROMATIUM
(not adjusted for control results)

LIGHT INTENSITY (Foot-candles)	THIOPEDIA Cell N (mg/l)	CHROMATIUM Cell N (mg/l)
40	26	32
80	39	44
120	45	55
160	54	57
200	57	53

If the preceding data, together with the data of the control cultures, are considered, somewhat different results are obtained. Not all cultures were prepared from the same batch of medium. All cultures for the 40 foot-candle series were from one batch, cultures of the 80 foot-candle and 120 foot-candle series were from another batch and cultures of the 160 foot-candle and 200 foot-candle series were from a third batch of medium.

It is noticed in Table V that there is quite a variation in results of the control cultures. However, this variation is from one batch of media to another, and the results of any one batch are quite consistent. Also, the variation of the Thiopedia controls is consistent with that of Chromatium controls. This variation may be due to what we can call a medium factor.

If all Thiopedia controls are equalized to a value of 53 mg/l and the Chromatium controls are equalized to 55 mg/l and the respective test cultures are adjusted in the same proportions, a set of curves as in Figure 18 results.

The curves show a decline of growth at high light intensities. This is not to say there definitely is a reduction in growth due to increased light intensity, but that the possibility of growth inhibition caused by excessive light intensity does exist.

Table V

EFFECTS OF VARYING LIGHT INTENSITY ON GROWTH OF
THIOPEDIA AND CHROMATIUM (adjusted for Control results)

LIGHT INTENSITY (Foot-candles)	THIOPEDIA			CHROMATIUM		
	Culture Cell N (mg/l)	Control Cell N (mg/l)	Culture Cell N Adjusted to Control Value of 53 mg/l (mg/l)	Culture Cell N (mg/l)	Control Cell N (mg/l)	Culture Cell N Adjusted to Control Value of 55 mg/l (mg/l)
40	26	40	35	33	53	34
80	39	29	55	44	47	51
120	45	29	82	54	47	63
160	54	53	54	57	55	57
200	57	53	57	53	55	53

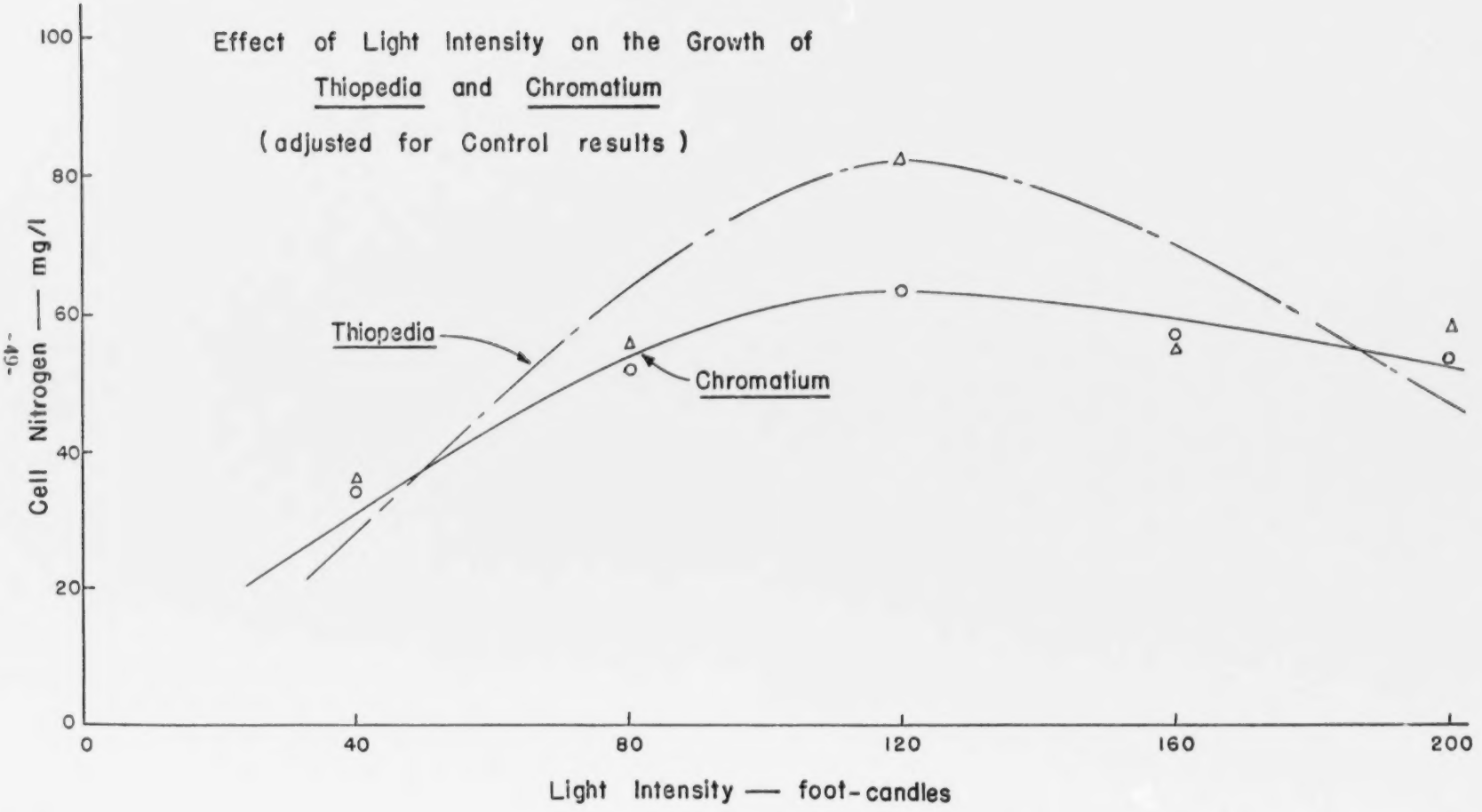
Effect of Light Intensity on the Growth of
Thiopedia and Chromatium
(adjusted for Control results)
Thiopedia
Chromatium
Cell Nitrogen — mg/l
Light Intensity — foot-candles
0
20
40
60
80
100
0
40
80
120
160
200

Fig. 18

Phase Four

It has been previously stated that the control of oxygen content was quite difficult and once the culture bottles were sealed it was impossible to control. Initial dissolved oxygen concentrations, in mg/l, used were 0.4, 1.0, 1.5, 3.0 and 4.0.

After a period in excess of two weeks all culture bottles had growth. Those containing Na_2S reached their maximum visual growth within three days and were very deep red in color. The remaining cultures began growing as the incubation period continued until all cultures showed growth. From visual observation, the growth in the cultures which initially contained dissolved oxygen was not as intense as the culture containing Na_2S. Also, there was no particular sequence to the progression of growth with respect to initial oxygen concentration. Cell nitrogen determinations were not made on this phase as it was felt they would be of no value.

The results appear to verify the theory that these organisms are microaerophils. However, it is still difficult to say definitely that the lack of dense growth is due to the presence of dissolved oxygen. Sodium sulfide, which was absent from the medium, may be a necessary nutrient in conjunction with the other ingredients.

Conclusions

Although sodium thiosulfate does not appear to be a critical growth factor above 500 ppm, the results have shown that growth increases to a maximum value in the vicinity of 6,000 ppm for Chromatium, and growth of Thiopedia increased with each increase in $Na_2S_2O_3 \cdot 5H_2O$, although the growth curve does level off at the higher concentrations. This is an extremely high concentration of this reduced sulfur compound to be present in most natural waste effluents. Since it was shown that Na_2S alone would not support growth, the availability of other reduced sulfur compounds or organic intermediates may be a limiting factor. This being the case, it is quite likely that this method of waste stabilization would not be possible with certain effluents.

Temperature is quite critical, and the optimum value appears to be in the range of 25°C to 35°C, with a rapid decline in growth outside this range. Therefore, it is concluded that for the most efficient use of this method of treatment there are some geographical limitations. For year-around use, this method would be limited to areas with consistently warm climates. There is the possibility that other species of these bacteria could exist at temperatures above or below those found to be optimum for the two species studied in this investigation (33, pp. 147-149).

Light intensity is not as critical as the other factors studied, but better rate of growth is achieved at higher intensities. There is a

possibility of growth inhibition at higher light intensities. In satisfying the temperature requirements of a warm climate an environment consisting of relatively high natural light intensities would be achieved and growth of the organisms would be enhanced.

It is difficult to make any conclusions on the effect of dissolved oxygen alone on the growth of these organisms. At best, it seems to be verified that the particular species studied are microaerophils rather than strict anaerobes. It has been noticed that the greatest growth density of Chromatium occurred at the interface where the dissolved oxygen curve and the H_2S curve joined at approximately zero values for each in Beloved Lake, Russia (33, p. 147). This suggests that certain species of these organisms do flourish in environments containing a small quantity of dissolved oxygen along with a constant supply of sulfide compounds. Also, dense populations of Chromatium along with other bacteria have been found in Lunzer Mittersee in stratified layers where the dissolved oxygen content amounted to 3-4 mg/l. This further substantiates the idea that these organisms are not strict anaerobes but may be designated as faculative oligoaerobes (33, p. 148).

III. FIELD STUDY

STRATIFICATION IN A WASTE TREATMENT POND

Ponds have become a popular means of treating wastes where land is relatively cheap because of the ease with which they can be constructed and operated. Although such ponds frequently do an adequate job of waste treatment, in many instances better design and better operating procedures would improve the efficiency of waste treatment. More information on the characteristics of the water in ponds is needed to decide what design and operating procedure are best.

It is well known that quite shallow ponds can become thermally stratified during calm, clear weather. Waste treatment ponds would be expected to stratify more frequently than other ponds, because they are typically turbid from algal blooms, and high turbidity favors stratification.

Thermal stratification inhibits mixing of the upper layers of water with the lower because of the difference in their density. This inhibition of mixing tends to bring about a stratification in other chemical and physical characteristics of the water, including oxygen, pH, alkalinity, and conductivity. Of these, oxygen has the most effect on the efficiency of waste treatment. In a stratified pond photosynthesis and aerobic decomposition occur in the upper stratum and anaerobic decomposition in the lower stratum of water.

Oswald (28) and Cooper, Oswald, and Bronson (9) state that most waste treatment ponds are stratified ("facultative"), but they do not present any supporting data. Indeed, there is only one previous study of stratification in a waste treatment pond, that by Bartsch and Allum (2). To obtain more information on stratification in temperature and oxygen in a waste treatment pond, a small pond near Pullman, Washington, was studied intensively from May through September, 1965. Temperatures were measured on 47 afternoons and on two 24-hour series. Oxygen samples were taken somewhat less frequently.

The pond receives wastes from an establishment that raises white rats for sale to various schools and laboratories in the Pacific Northwest. From 6,000 to 8,000 animals are maintained. Once a day the feces are washed from under the cages with an average of 2.5 cu m of water. The wastes drain into a small pond with a volume of 31 cu m, 12 times that of the

average inflow, where they are given primary treatment. The effluent then flows into the pond studied. Before primary treatment, BOD of the waste was 1700 mg/l; after this treatment BOD was 135 to 486 mg/l. It was not possible to measure accurately the flow of the primary effluent into the rat pond, so loading cannot be measured accurately. During May and September, there was some outflow from the rat pond, but most of the summer there was none. The water level varied less than 5 cm (except for two days when the dam leaked.)

From late March to 4 June, a bloom of the green alga, Chlamydomonas, was present. About 10 May, a bloom of the purple sulfur bacterium, Thiopedia, also developed. By 21 June these organisms had been displaced by a bloom of the green alga, Chlorella, which persisted throughout the remainder of the study period. Chlorella averaged about 10,000,000 cells/ml, and Euglena one percent or less of that number. The coefficient of light extinction was determined with a spectrophotometer and from this the depth at which one percent of the surface light remained was calculated. (This depth is commonly accepted as the lower boundary of the euphotic zone.) The depth of the euphotic zone was consistently less than 2 cm -- a remarkably low value.

Temperature was measured with a Whitney thermistor, which had a scale graduated to 0.1 ^{o}C. Water was collected with a special sampler consisting of a plexiglas cylinder 60 cm long and 69 mm inside diameter, with side ports at 10, 30 and 50 cm. After immersion, the bottom of the cylinder was closed with a plexiglas disc attached to a long handle. Samples were taken in 21-ml vials having conical screwcaps. With this sampler it was possible to obtain samples from precise depths, with little disturbance of the water. Surface samples were simply dipped out. The Alsterberg (azide) modification of the Winkler method was used to fix the samples of oxygen. All sampling was done from a dock to avoid disturbance of the water. The afternoon series were taken on 1330 hrs PDT $\pm$ 30 minutes.

Results

Temperature at 1330 hrs

In June, a representative month, a sharp thermal stratification was present on most days (Figure 19). The temperature at 1 cm exceeded 30 ^{o}C on six days. On all of these days the weather was calm, and clear to partly cloudy. Most of the temperature drop occurred in the upper 10 to 15 cm; sometimes the rate of drop was 1 ^{o}C/cm. On two days, 11 and 25 June, the isotherms were depressed 10 to 15 cm. On both of these days the weather was moderately breezy and partly cloudy. On 14 June, stratification was virtually absent. This was the coolest, windiest, and most heavily overcast day in the month.

A simple way to compare the degree of stratification is to use the drop in temperature between 1 cm and 50 cm (Table IX). The frequency of sharp stratification is evident; on three-fourths of the days the temperature dropped more than 10°C, and on only four days was the drop less than 5°C. In each month the maximum drop in temperature occurred on a calm, clear day. The minimum drop, when less than 3°C, was on overcast, windy days. In July, the minimum drop of 8.1°C occurred on a completely clear, but windy, day.

The temperature of the surface water tended to be higher and much more variable than that of the water at 30 cm and below (Figure 20). The temperature at 50 cm was usually about the same as at 30 cm; thus, on most days almost all of the drop in temperature occurred in the upper 30 cm of water.

Although the temperature drop was very pronounced, the pond was less resistant to mixing than the differences in temperatures imply, because of its small volume.

Diel Variations in Temperature

To observe diel (24 hr) changes, measurements of temperature were made at two-hour intervals for 24 hours on 28 and 29 July 1965, and for 26 hours on 23 and 24 September, 1965. The weather was calm and clear during both sampling series, except for a cloud from 0730 to 1130 hrs on 29 July. Both series showed similar changes, so only the data for September are presented (Figure 21). By 2130 hrs the pond was essentially uniform in temperature, and it remained so until between 0930 and 1130 hrs the next morning. Temperature variation was large at the surface and small at 50 cm; at 1 cm the range in temperature during 26 hrs was 17.1°C; at 10 cm 9.7°C; at 30 cm 2.8°C; and at 50 cm 1.9°C. There was a slight temperature inversion from 1930 to 0730 hrs.

Although the maximum temperatures on 23 and 24 September were within 0.5°C of each other, the 12 C isotherm was 12 to 14 cm lower on 23 September than on 24 September, even though the weather was clear and calm on both days at the time of sampling. The explanation for this seeming anomaly is that prior to sampling in the 23rd the sky had been partly cloudy and a mild (15 km/hr) breeze had been blowing while on the 24th the weather was completely clear and calm during the entire 24 hours preceding the temperature series at 1330 and 1530 hrs. The minimum of 9.9°C at 50 cm at 1530 hrs is curious; we can think of no explanation.

Oxygen Concentrations at 1330 hrs

To illustrate the effects of weather on the concentration of dissolved oxygen, seven dates have been selected that represent contrasting

types of weather (Table VII).

On 5 July the weather was completely calm and clear (and hot). The contrast in DO concentration at the surface and at 5 cm is remarkable. On 24 September, also a perfectly clear and calm day, the surface concentration was even higher than on 5 July, although of the same order of magnitude. No sample was taken at 5 cm, but the relatively high concentration at 10 cm, one-tenth that at the surface indicates that the stratification of DO was not nearly as sharp on this date. The difference in oxygen distribution on these two days was probably caused by the difference in temperatures. On 5 July the mean temperature was 19.4°C; and 24 September it was 12.4°C. At 1 and 10 cm, the temperatures in September were more than 10° lower than in July. Since the rate of oxygen utilization increases roughly two or three times for each 10°C rise in temperature (van't Hoff's rule), there is no doubt that the rate of oxygen utilization was considerably higher on 5 July than on 24 September.

TABLE VI -- Temperature Drop in Upper 50 cm at 1330 hrs in the Rat Pond

Month	No. of days	5°	5° 10°	10°	Minimum	Maximum
May*	11	1	2	8	2.9°C	17.8°C
June	12	1	1	10	2.0	20.5
July	11	0	1	10	8.1	20.1
August	7	1	2	4	1.5	15.1
September	6	1	1	4	0.8	14.6
	47	4	7	36		

* Temperature differences in May are between 4 cm and 50 cm, with one exception.

TABLE VI-- Vertical Distribution of Oxygen in the Rat Pond at 1330 hrs on Seven Dates

Date	Weather	Surface		5 cm		10 cm		30 cm	
		mg/l	% Sat.	mg/l	% Sat.	mg/l	% Sat.	mg/l	% Sat.
5 Jul	Calm Clear	25.4	317	0.3	3	0		0	
24 Sep	Calm Clear	29.6	396			3.4	36	0	
8 Jul	Windy Clear	13.9	185			7.8	105	0	
25 Jun	Windy Pt. Cldy	6.1	82	3.6	50	2.6	35		
19 Aug	Calm Ov-cast	8.0	108	0		0		0	
12 Aug	Windy Ov-cast	0		0		0		0	
3 Sep	Windy Ov-cast	2.0	22			0.7	8	0.6*	6

* Sample from 50 cm.

TABLE VII-- Morphometry of Ponds

Pond	Area (sp m)	Depth (cm)	Volume (cu m)	Relative depth (%)
Rat	252	100	138	5.6
Dairy #3	885	130	852	3.9
Dairy #4	3106	133	2827	2.1
Modal Texas Pond	8100	91	--	0.6

FIGURE LEGENDS

FIGURE 19 -- Time-depth diagram of temperatures at 1330 hrs during June in the rat pond. Measurements were made on the dates and at the depths shown.

FIGURE 20 -- Summary of temperatures at four depths at 1330 hrs in the rat pond.

Figure Legends, Cont.

FIGURE 21 -- Time-depth diagram of diel changes in temperature on 23 and 24 September 1965 in the rat pond. Measurements were made on the dates and at the depths shown. Temperatures at 1 cm and 50 cm are also shown.

FIGURE 22 -- Variations in oxygen concentrations at the surface and at 10 cm in the rat pond on 24 September 1965. The vertical bars indicate the deviation of duplicate samples from the mean.

The weather was completely clear on 8 July also, but it was windy, with fresh gusts of perhaps 30 km/hr. The surface water had a substantial supersaturation of DO, but it was not nearly as high as on the calm and clear days. At 10 cm the concentration of DO was the highest found at that depth on any date. Thus, the clear sky and plentiful sunlight caused a high production of oxygen, and the wind distributed the oxygen to a greater depth than usual. Doubtless more oxygen was lost to the atmosphere from the surface of the water because of the wind-created turbulence. Despite the relatively high concentration of oxygen at 10 cm, no oxygen was found at 30 cm. This illustrates the importance of thermal stratification in controlling the distribution of oxygen.

Two factors acted to reduce the concentration of DO at each depth on 25 June: partial cloudiness, which reduced the total amount of isolation and hence the amount of oxygen produced by algal photosynthesis; and windiness, which mixed the oxygen produced with a larger volume of water having a high oxygen demand. Hence it is not surprising that the oxygen was not even at saturation value at the surface. At 10 cm DO was relatively high, when compared to the surface concentration on this date, demonstrating the effect of the wind in carrying oxygen downward.

On 19 August, the weather was dead calm, heavily overcast (no shadows), and sultry. Because of the overcast, oxygen production must have been relatively low. Nevertheless, there was a slight supersaturation of oxygen at the surface, because there was so little mixing of the surface water with water having a high oxygen demand. The lack of mixing also caused anaerobic conditions even at 5 cm.

On the two days which were windy and overcast, 12 August and 3 September, oxygen production must have been low because of the overcast, and the oxygen produced was mixed with the entire volume of water in the pond. The absence of DO in August and its presence at all depths in September indicate that the oxygen balance, the ratio of oxygen demand to oxygen available, was different on the two dates. Doubtless, oxygen demand was higher in August than in September because the temperature was higher. On 12 August, the mean temperature was 19.4°C; while on 3 September the mean temperature was 13.7°C, almost 6° less. Evidently, the oxygen demand of the entire pond in August was high enough to use up all oxygen produced, while

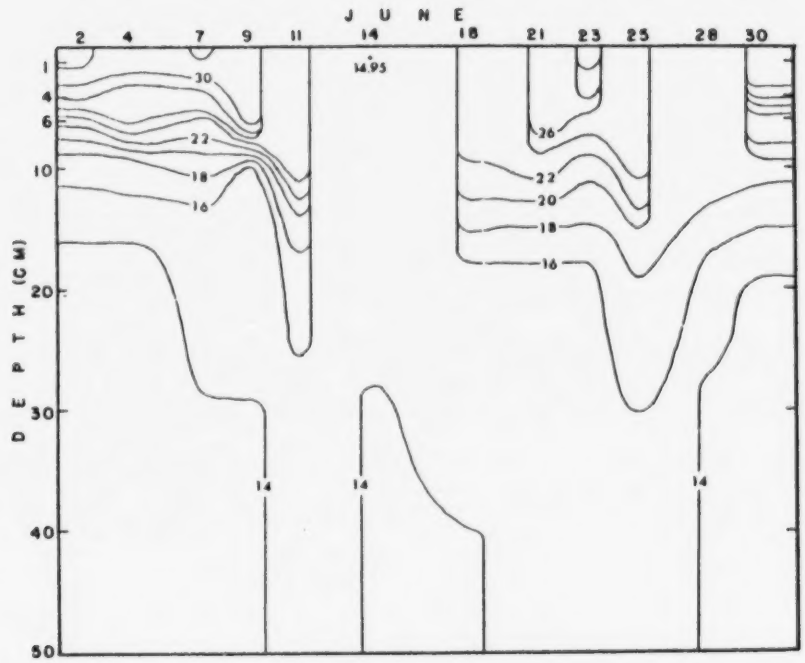

Fig. 19 Legend located on page 56.

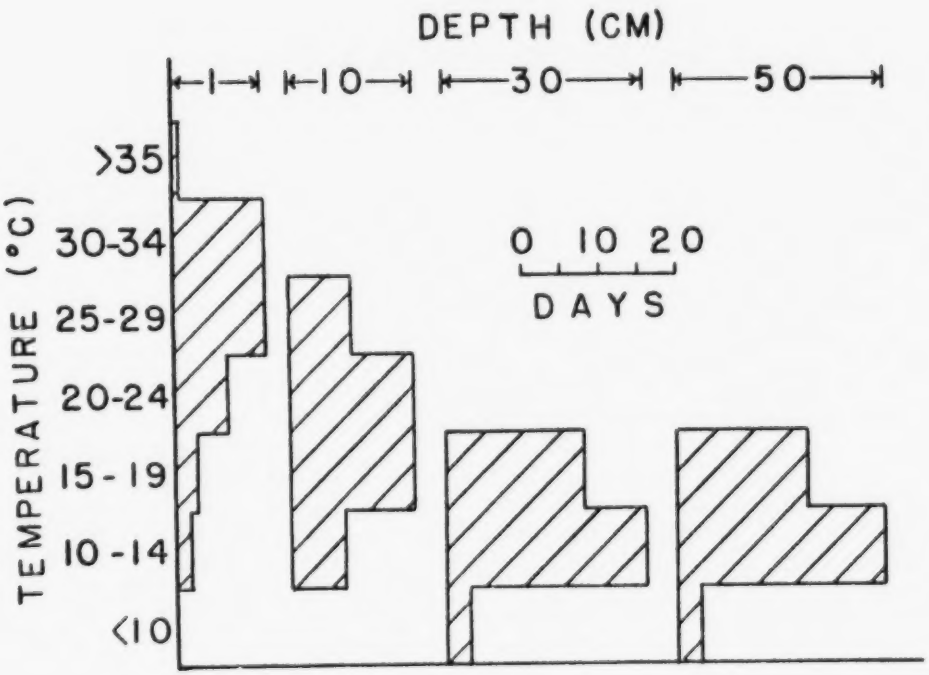

Fig. 20. Legend located on page 56.

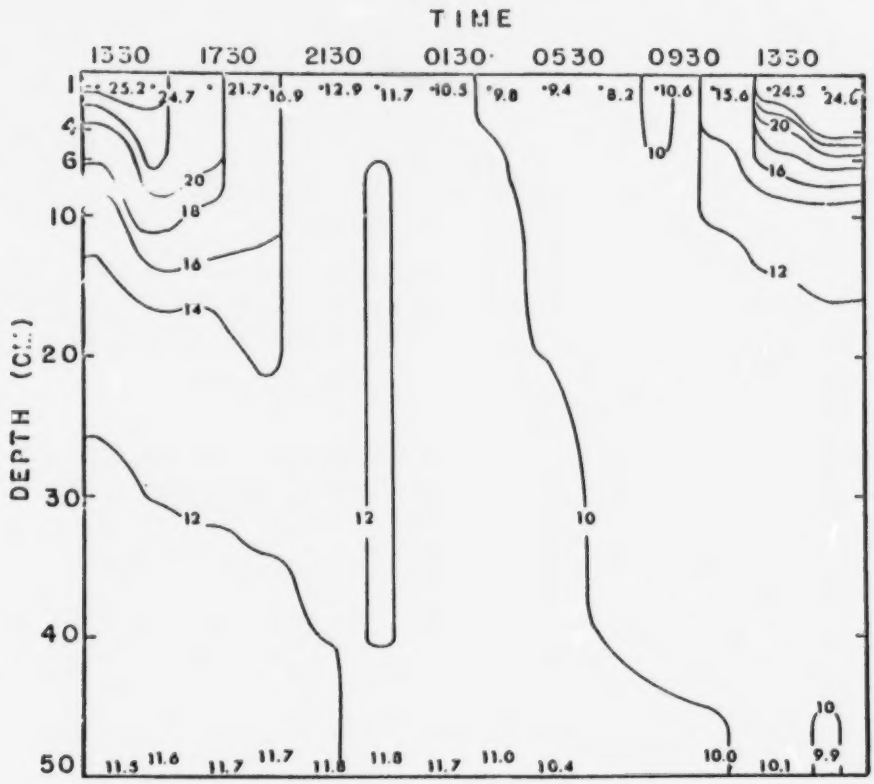

Fig. 21 Legend located on page 57.

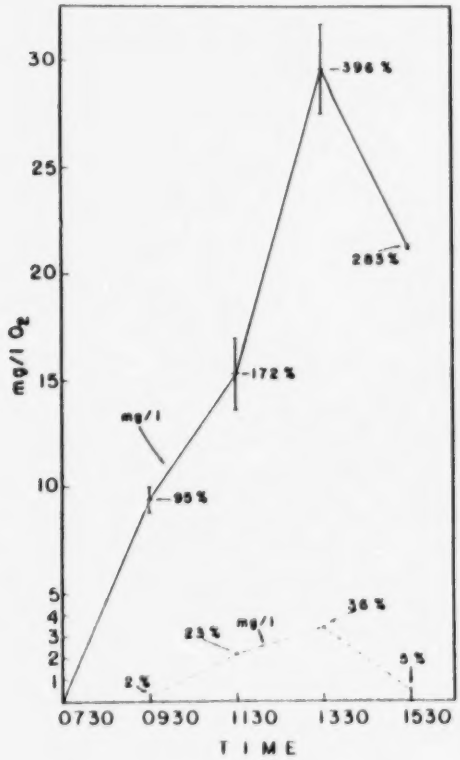

Fig. 22 Legend located on page 57.

in September it was not. Consequently, only on 3 September was oxygen present below 30 cm.

Meteorological conditions are, of course, often more variable than on the dates given in Table X. Nevertheless, supersaturation at the surface was the rule; more than 150 percent saturation occurred at the surface on 16 of 33 days.

Diel Variations in Oxygen Concentration

The diel variation in oxygen concentration was very large at the surface on clear, calm days (Figure 22); in less than five hours the oxygen concentration increased from zero to almost four times the saturation value. At a depth of only 10 cm, the pattern of change was similar to that at the surface, but the magnitude of change was much smaller. It is not clear why DO decreased between 1330 and 1530 hrs.

There is no doubt that the rat pond became entirely anaerobic each night, although just when it became anaerobic is not known. A sample taken at 1940 hrs on 23 September showed a saturation of 39 percent -- one-fifth of the concentration at 1330 hrs. During this same interval, the DO at 10 cm increased from 7 percent to 15 percent of saturation due to the sinking of the oxygen-rich surface water as it cooled.

Discussion

The present investigation is the only one to document the effect of weather on stratification of temperature and oxygen in a waste treatment pond. On most days the rat pond, despite its shallow depth, was stratified; on calm, clear days the stratification was sharper than that reported for any other pond of comparable size, but on heavily overcast, windy days stratification was virtually absent. Each night stratification disappeared, even when stratification had been very sharp during the day and there was no wind.

These observations could have been predicted. Thermal stratification develops when the radiant energy of the sun is absorbed in the upper layer of water, making this layer warmer and lighter than the water below. The radiant energy must be absorbed before reaching the bottom of the pond or lake, otherwise there can be no differential warming. Thus stratification is more likely to develop in deep lakes and turbid ponds than in shallow lakes and ponds. The wind mixes surface water with the water below eliminating stratification. Thus, stratification is more likely to develop on calm days and in bodies of water protected from the wind. The morphometry (size and shape) of a body of water affects stratification. A small volume of water warms and cools more quickly than a large volume of water; hence, ponds typically have a transitory stratification. A large area gives the wind a greater sweep, and hence permits it to become stronger than it can in a small area. The effect of the wind on stratification also depends on the depth, because a greater volume of water must be mixed in a deep body of water than in a shallow body of water.

Because of the importance of the relationship between area and depth for stratification, a parameter that relates the two is desirable. The relative depth is such a parameter. Hutchinson (21) defines the relative depth as:

$$z_r = 50 z_m / A \qquad (1)$$

where z_r = relative depth (as a percentage)

z_m = maximum depth (in meters)

A = surface area (in square meters)

Relative depth represents the maximum depth as a percentage of the diameter of a circle that is the same area as the pond. For a pond (or lake) of a given area, the higher the relative depth, the more likely is thermal stratification to develop.

How typical are the results on the rat pond? It is difficult to say, because so few data on other ponds are available. In the Kadoka, South Dakota, pond studied by Bartsch and Allum (2) the temperature dropped 4.0°C in 50 cm, and DO varied from a maximum of 30 mg/l to zero at 90 cm. Unfortunately, no information on weather was provided, so a close comparison with the rat pond is not possible. Nine series of measurements were made on two ponds near Pullman that receive wastes from dairy cattle. A temperature drop of more than 5°C in 50 cm occurred on every day, and on a clear, calm day the maximum temperature drops of 15.3°C in Dairy Pond #3 and 16.9°C in Dairy Pond #4 occurred.

No other measurements of stratification in waste treatment ponds have been made, but it is useful to compare the two factors affecting stratification, namely, depth of light penetration and morphometry. Bartsch and Allum (2) and Copeland, Minter, and Corris (10) reported euphotic zones as thin as 10 cm and 46 cm, respectively. Quite likely the very limited light penetration in the rat pond is not usual, however, as the two dairy ponds had a similar depth of euphotic zone. Waste-treatment ponds vary widely in size and shape; in Table XI are given the area, depth and relative depth of the average (modal) depth of the rat pond and of the dairy ponds was considerably higher than that of the average pond.

The absence of stratification at night and on days that are cloudy and windy is highly significant for the operation of the rat pond. Whether it became completely anaerobic or completely aerobic depended on the oxygen balance. Complete anaerobiosis is preferable, so that populations of obligate anaerobic bacteria will be maintained. The methane bacteria are obligate anaerobes; since from 90 to 95 percent of the BOD of added waste is transformed into methane and other gases in a properly operated pond (Cooper et al. 9), it is desirable to maintain a population of these bacteria. At night anaerobiosis can be achieved more easily, because there

is no photosynthetic production of oxygen; during the day photosynthesis might cause the oxygen available to exceed the oxygen demand, as happened on 3 September (Table X). Cool weather reduced the oxygen demand below the oxygen available on that date, so heavier loading in cool weather would be desirable.

Permanent stratification would assure the maintenance of anerobic conditions in the lower waters, but it would probably be impracticable to construct a pond sufficiently large and deep to remain stratified at night and in all kinds of weather. However, the frequency of stratification probably could be raised by designing ponds with a high relative depth. Thus the rat pond and the two dairy ponds were usually stratified and had high relative depths compared to the modal Texas pond (Table XI). A relative depth of at least two percent is suggested as a guideline. When this guideline is used, a pond 1,000 sq m in area should be at least 0.7 m deep, one 5,000 sq m in area 1.6 m deep, and a pond 10,000 sq m in area 2.2 m deep.

Finally, this study demonstrates the need to be precise about "surface" measurements of temperature and oxygen, and the need to consider the weather when making those measurements.

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Sulfide Uptake by the Purple Sulfur Bacterium Chromatium, by David W. Alton.

The Effect of Certain Ecological Factors on the Growth of Thiorhodaceae, by J. M. Anglea.

Growth of Thiopedia and Chromatium Organisms on Various Substrates, by Ronald J. Thompson.

A limited number of copies of these papers are available for distribution and can be obtained from the Sanitary Engineering Section, Division of Industrial Research, Washington State University.

APPENDIX

SAMPLE RUN #1

Sample	Time	Filter CPM	CPM-BG	Filtrate CPM(.2ml)	CPM-BG
1 (1.5)ml	0	4048	4021	4369	4342
2 "	20 sec	4395	4368	NT	NT
3 "	40 "	4104	4077	NT	NT
4 "	60 "	4987	4960	NT	NT
5 "	80 "	5447	5420	NT	NT
6 "	100 "	5299	5272	NT	NT
7 "	2 min	4976	4949	NT	NT
8 "	5 "	6320	6293	NT	NT
9 "	10 "	6779	6752	4934	4907
10 "	30 "	10,909	10,882	8289	8262
11 "	60 "	13,893	13,886	8410	8383
12 "	120 "	16,769	16,742	11,108	11,081
13 "	180 "	19,894	19,867	7,335	7,308
14 "	240 "	19,317	19,290	12,941	12,914
15 "	300 "	20,756	20,729	7,249	7,222
16 "	360 "	20,995	20,968	8,383	8,356

BG = 27 CPM

ISOTOPE = 2 mls.

*NT = Not Tested

**P.C. = 10 Units at "0 Time"

**P.C. = 10 Units at 360 Min.

**P.C. = Photoelectric Colorimeter Reading of Cell Concentration

SAMPLE RUN #2

Sample	Time	Filter CPM	CPM-BG	Filtrate CPM(.2ml)	CPM-BG
1 1.5 ml	0	22,538	7487	45,141	15,015
2 "	39 sec	22,438	7444	45,831	15,178
*3&4 "	74 "	42,002	13,968	55,774	18,559
5 "	80 "	27,280	9,061	46,345	15,416
6 "	100 "	29,868	9,921	54,064	17,989
7 "	2 min	29,489	9,798	47,085	15,663
8 "	5 "	35,354	11,753	44,326	14,743
9 "	11 "	42,370	14,091	46,604	15,503
10 "	30 "	49,820	16,575	44,609	14,837
11 "	60 "	55,234	18,713	46,304	15,403
12 "	120 "	55,162	18,355	52,637	17,514
13 "	184 "	58,364	19,423	53,933	17,946
14 "	240 "	47,074	15,659	69,201	23,035
15 "	300 "	64,290	21,298	56,275	18,726
16 "	360 "	51,051	16,985	68,675	22,859

BG = 32 CPM

ISOTOPE = 2 mls.

**P.C. = 29 Units at "O Time"

**P.C. = 30 Units at 360 min.

* = Samples 3 & 4 on same filter

**P.C. = Photoelectric Colorimeter Reading of Cell Concentration

SAMPLE RUN #3

Sample	Time	Filter CP3 min.	CPM-BG	Filtrate CP3 min.(.1ml)	CPM-BG
1 1.5 ml	0	32,363	10,756	48,954	16,286
2 "	30 sec	36,988	12,297	41,151	13,685
3 "	1 min	35,433	11,779	38,192	12,699
4 "	1.5 "	39,710	13,205	41,011	13,638
5 "	2 "	58,120	19,341	42,908	14,271
6 "	2.5 "	61,723	20,542	57,026	18,977
7 "	3 "	50,234	16,713	45,137	15,014
8 "	5 "	61,297	20,400	57,637	19,180
9 "	10 "	55,085	18,330	46,553	15,486
*10 "	12 "	755,196	251,700	Total	Total
11 "	30 "	62,464	20,789	66,108	22,004
***12 "	75 "	102,871	34,258	51,896	17,267
13 "	150 "	78,880	26,261	50,799	16,901
14 "	210 "	86,266	28,723	53,046	17,650
15 "	240 "	88,370	29,425	51,920	17,274
16 "	300 "	122,415	40,773	49,756	16,533
*17 "	301 "	814,361	204,775	Total	Total
18 "	360 "	161,796	53,900	50,911	16,938

BG = 32 CPM

ISOTOPE = 5 mls.

* = Total Sample of Cells and Medium (1.5 ml)

**P.C. = 29 Units at "0 Time"

**P.C. = 31 Units at 360 min.

**P.C. = Photoelectric Colorimeter Reading of Cell Concentration

*** = Sample with Rubber on Filter

SAMPLE RUN #4 (Cold Room)

Sample	Time	Filter CP3 min.	CPM-BG
1 1.5 ml.	0	75,395	25,100
2 "	20 sec	61,458	20,454
3 "	40 "	101,050	33,651
4 "	60 "	71,351	23,752
5 "	80 "	65,719	21,874
6 "	100 "	65,419	21,774
7 "	2 min	61,988	20,631
8 "	5 "	70,230	23,378
9 "	10 "	78,388	26,097
10 "	30 "	87,399	29,101
11 "	1 hr.	95,996	31,967

BG = 32 CPM

ISOTOPE = 5 mls.

**P.C. = 30 Units at "0 Time"

**P.C. = 30 Units at 1 hour

**P.C. = Photoelectric Colorimeter Reading of Cell Concentration

SAMPLE RUN #5

Sample	Time	Filter CP3 min.	CPM-BG	Filtrate CP3 min.(.1ml)	CPM-BG
1 1.5 ml	0	55,986	18,630	45,498	15,134
2 "	29 sec.	59,665	19,856	58,493	16,132
3 "	43 "	59,821	19,908	64,062	21,322
4 "	60 "	70,244	23,383	47,325	15,743
5 "	80 "	65,440	21,781	54,275	14,726
6 "	100 "	67,168	22,357	57,100	14,001
7 "	2 min.	71,440	23,781	50,350	16,751
8 "	5 "	66,956	22,287	58,190	19,365
9 "	10 "	95,682	31,862	64,198	21,367
10 "	30 "	118,874	39,593	64,661	21,522
11 "	62 "	105,029	34,978	58,901	19,602
12 "	120 "	171,113	57,006	56,614	18,839
13 "	182 "	111,265	37,056	59,305	19,736
14 "	240 "	114,027	37,977	64,107	21,337

BG = 32 CPM

ISOTOPE = 5 mls.

**P.C. = 34 Units at "0 Time"

**P.C. = 37 Units at 240 min.

**P.C. = Photoelectric Colorimeter Reading of Cell Concentration

SAMPLE RUN #6 (Cold Room)

Sample		Time	Filter CP3 min.	CPM-BG
***1	1.5 ml.	0	64,515	21,473
2	"	.5 min	46,900	15,601
3	"	1 "	42,864	14,256
*4	"	2.5 "	884,430	294,778
5	"	3 "	44,075	14,660
6	"	5 "	45,442	15,115
7	"	10 "	47,220	15,708
***8	"	30 "	76,603	25,502
***9	"	1 hr.	80,973	26,959
10	"	2 "	60,697	20,200

BG = 32 CPM

ISOTOPE = 5 mls.

* = Total Sample of Cells and Medium (1.5 ml)

**P.C. = 28 Units at "0 Time"

**P.C. = 28 Units at "120 min"

*** = Samples with Rubber on Filter